Genetics of susceptibility to tuberculosis

Thesis

GENETICS OF SUSCEPTIBILITY TO TUBERCULOSIS

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This thesis is dedicated to the glory of God Almighty and to collaborative medical research.

ABSTRACT

Convincing evidence that activated macrophages play a critical role in control of mycobacterial diseases has been clearly established from animal and in-vitro studies. Macrophages produce a variety of molecules upon appropriate stimulation, which act in concert towards eventual killing of bacteria. People with sub-optimal macrophage activation are more susceptible to infection with intracellular pathogens. My project aims to answer two questions relating to genetic regulation of macrophage activation in tuberculosis: do macrophage genes regulate microbial-induced responses and do macrophage genes influence susceptibility to tuberculosis? A whole blood assay was used to investigate IFN-γ responsiveness in healthy individuals and those who develop tuberculosis in The Gambia. Cytokine responses to lipopolysaccaride (LPS), Lipoarabinomanan (LAM) and the enhancing effect of IFN-γ on these stimulants were measured. LPS induced IL-10 levels was higher in recovered TB cases than in controls ($p=0.02$). LPS and LAM induced cytokines were highly correlated ($p<0.0001$) similarly, levels of IL-1β and TNF were highly correlated ($P<0.0001$). Ten new polymorphisms were detected by sequencing specific regions within the promoter of *IFNG* and *IFNGR1* genes. One, a double deletion of TT in the promoter of *IFNGR1*, abolishes a GAS binding site at position –470 and another, a C/T transition, is close to a putative NF-kappa β binding site at position -56 in the *IFNGR1* gene (positions are relative to the transcription start site). These along with published polymorphisms at some macrophage candidate gene loci were genotyped. Comparisons were made to determine whether different alleles at candidate gene loci influence macrophage cytokine responses. *TNFA*-863, *LTA NcoI, IL1RN* and *NRAMP1* (469+14) polymorphisms were shown to influence macrophage cytokine levels significantly. *TNFA-863* was associated with LPS induced TNF ($P < 0.05$), *LTA* was associated with

LAM and LPS induced TNF and IL-β levels ($p < 0.01$). NRAMP1 (469+14) was associated with LAM induced IL-10 ($P<0.01$) and *IL1RN* was associated with LAM and LPS induced IL-10 ($P<0.05$).

Alleles 1 (G) of *TNFA*–308, 2 (A) of *TNFA*-238, 1 (T) of *IL1B*-511 and 2 (ddel/T) of *IFNGR1* were significantly associated with TB in the panel of samples studied. For the microsatellite markers, allele 5 of *IL9* (TG)n repeat in intron 4 and allele 3 of the Z DNA promoter polymorphism *NRAMP1*, were significantly associated with TB.

NRAMP1 INT4 variant was significantly associated with both TB and LAM induced IL-10 secretion.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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LIST OF ABBREVIATIONS

A	adenine
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cells
AT	annealing temperature during PCR
B	bonferroni adjustment
BCG	bacille Calmette-Guerin
bp	base pair
C	cytosine
CD	cluster differentiation
°C	Celsius
cM	centiMorgan
CMI	cell mediated immunity
DC	dendritic cells
ddel TT	double deletion of thymine
DF	degrees of freedom
DN	double negative T cells
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
G	guanine
GAS	gamma activated site
GAF	gamma activation factor
GM-CSF	granulocyte monocyte colony stimulating factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFNγ	interferon gamma
IFNG	interferon gamma gene
IFNγR1&2	interferon gamma receptor ligand and signal transducing protein
IFNGR1	interferon gamma receptor ligand binding chain gene
IFNGR2	interferon gamma receptor signal transducing chain gene
IL-1β	interleukin 1 beta
IL1B	interleukin 1 beta gene
IL-1RA	interlekin 1 receptor antagonist
IL1RN	interleukin 1 receptor antagonist gene
IL-3, 4,5 etc	interleukin 3,4,5 etc
iNOS	inducible nitric oxide synthase
IRF-1	interferon regulatory factor-1
LAM	lipoarabinomannan
LPS	lipopolysaccahride
MIM	mendelian inheritance in man
M-H	Mantel Haenszel test
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NF-kβ	nuclear factor kappa beta
ng	nanogram
NK	natural killer cells
NO	nitric oxide
NOS2A	inducible nitric oxide gene
Nramp1	murine natural resistance associated macrophage protein

NRAMP1	human natural resistance associated macrophage protein
NS	no significant association between rows and columns
NTM	non tuberculous mycobacteria
OD	optical density
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cell
PCD	programmed cell death
PNG	polymorphnuclear granulocyte
PPD	purified protein derivative
PRR	pattern recognition receptors
P-value	conditional probability of observed data occurring assuming null hypothesis is true
QTL	quantitative trait locus
RFLP	restriction fragment length polymorphism
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
S	significant association between rows and columns
SCYA	small inducible chemokines
SE	standard error of mean
SNP	single nucleotide polymorphism
STAT	signal transducers and activators of transcription
T	thymine
TAP	transporter associated with antigen presentation
TB	tuberculosis
TCR	T cell receptor
Th	T-helper cell
TLR	toll-like receptor
TNF	tumour necrosis alpha
TNFA	tumour necrosis factor alpha gene
TNFa	tumour necrosis alpha microsatellite a
UTR	untranslated region
VNTR	variable number tandem repeat
WHO	World Health Organisation
χ^2	chi square
Z-DNA	Structural conformation of a polymorphic DNA with enhancer activity

CHAPTER 1

INTRODUCTION

1.1 Tuberculosis

1.1.1 Tuberculosis: an overview

Tuberculosis (TB) is currently the world's single leading infectious cause of adult deaths. In 1990, it was estimated that about 1,700 million people, a third of the world's population, were infected with *Mycobacterium tuberculosis* (Raviglione *et al.*, 1997). In 1993 the World Health Organization (WHO) declared TB to be a global emergency (Reichman, 1996). A recent WHO report estimated that in 1998 there were 8 million new cases of clinical tuberculosis and 1.9 million deaths from the disease. It is expected to kill a hundred million more over the next thirty years. Although BCG vaccine constitutes an integral part of the TB control and Expanded Programme of Immunisation (EPI) in most developing countries, its efficacy varies between populations from 0-80% (Fine, 1989). Current treatment relies on a combination of drugs acting against the micro-organism, but unfortunately about 30% of organisms are now resistant to first-line drugs (Jacobs, 1994). Also, treatment must continue for 6-9 months which is often not practical, especially in developing countries, and results in poor compliance. In fact, because of extensive poverty most control programmes have been below the required standard, resulting in many clear failures of treatment. Although the overall proportion of infected people is similar in industrialised and developing countries, the age of infection differs: 80% of infected individuals in industrialised countries are aged 50 years or more while 75% of those in developing countries are less than 50 years old (Harris, 1997). The epidemic is increasing in both industrialised and developing countries but still has not reached its peak in most countries of Africa. Sub-Saharan Africa has the greatest burden of morbidity and mortality from TB (Dolin, 1994), a large proportion of which is now associated with co-infection with the human immunodeficiency virus (HIV; Hawken *et al.*, 1993). The increase has mainly occurred in childbearing and economically active groups. An increase

in HIV related childhood TB has been documented from most African countries such as Zambia and Côte d'Ivoire (Harris., 1997). Social factors such as population growth, smoking, alcoholism, over-crowding, war and natural disasters such as drought and famine create conditions that predispose to the spread of TB (Daniel *et al.,* 1994). Economic priorities and government policies in the worst affected areas preclude health-related expenditure. General lack of first line drugs for tuberculosis has resulted in drug misuse and therefore resistant organisms. The most cost effective control tool, prevention, would be aided by the development of a more effective vaccine. For this, an understanding of the host determinants of protective immunity is paramount.

Human pulmonary infection with *M. tuberculosis* results in parasitisation and activation of alveolar macrophages (Hirsch *et al.,* 1994). At the initial stage of infection, the only evidence of infection in most cases is the development of a positive tuberculin skin test (Hopewell, 1994). Within the first 2 years after infection only about 10% develop disease (Bloom and Small, 1998). For those who do, there are two main clinical syndromes. Miliary TB is the most serious form of the disease and occurs in children, immunosupressed individuals and patients with advanced pulmonary TB who have an ineffective immune response. It is characterised by hematogenous dissemination of large numbers of organisms throughout the body and severe disease that is almost invariably fatal if untreated. This form of the disease is characterised by a high frequency of negative tuberculin skin test and failure of T lymphocytes to respond to *M. tuberculosis* antigens (Uberoi *et al.,* 1975). At the other end of the disease spectrum is tuberculosis pleuritis where patients mount a strong immune response to infection reflected by resolution of pleuritis often without therapy. Between these extremes lie a number of other clinical phenotypes including bronchopulmonary TB, TB meningitis, pericarditis and osteomyelitis (Harris, 1997).

1.1.2 An historical perspective

In 1882 Robert Koch identified *M. tuberculosis* as the causative agent of TB (Grange and Bishop 1982). Tuberculosis is an ancient disease and the aetiologic agent probably preceded the development of man on earth. It is speculated that cattle were the source of human TB infection and that *M. tuberculosis* evolved from *M. bovis*, which has a broad host range, capable of infecting man and several other species. *M. tuberculosis* is pathogenic only to man (a point relevant to the use of animal models in TB research generally). The first human infections presumably were isolated events resulting from eating infected meat or drinking contaminated milk. As man started settling in villages and began cultivation in 7000 BC, and domestication of cattle swine and sheep occurred, TB probably occurred more frequently in man. Records dating back to the time of Hippocrates suggest that TB existed, and was referred to as phthisis, the Greek word for wasting. (Castiglioni, 1933). Signs of spinal tuberculosis were found in Neolithic skeletons and early Egyptian remains and clearly depicted in figurines and paintings throughout the era (Morse *et al.*, 1964). Examinations of prehistoric human skeletons in South America between 100 BC and 1300 AD revealed skeletal deformities compatible with TB.

TB became a major problem in the western world when the industrial revolution created crowded urban living conditions, favouring the spread of the bacillus. Oliver Wandall Holme's "white plague" (Dubos and Dubos, 1952) arose in the 1600's and peaked at the beginning of the nineteenth century when TB was the commonest cause of death in Europeans. The epidemic spread later to United States and then world-wide as infected Europeans migrated to more distant sites (Budd, 1867). In the 1880's when native Americans were forced to live in small fixed huts, an epidemic began. Contact with surrounding white settlers became frequent and people started living in crowded

communities. These conditions favoured the rapid spread of TB and death rates from TB increased rapidly: by 1886, the TB death rate was 10 times higher in the indigenous population than the Europeans.

As this was happening in America, TB was still practically unknown in most of North and sub-Saharan Africa. Notable scholars resident in different parts of Africa for decades did not record any TB in their communities. Black people taken to America were free of TB upon arrival but between 1822 and 1861 TB death rates gradually increased. Early reports of TB in Africans occurred following close contact with Europeans or Egyptians (Cummins, 1908). Healthy young Senegalese men recruited into the French Army during World War I died in great numbers as a result of first time exposure to the bacillus. TB was first reported in South Africa in 1908 in black men working in mines (Cummins, 1929). By 1967, Paviot reported endemicity of TB in several African countries including The Gambia (Paviot, 1967). The British Medical Research Council and East African Research Centres carried out large surveys in Kenya and Tanzania between 1964 and 1984. All together 8741 TB patients were studied; 90% had pulmonary disease, others had lymphadenopathy, bone or joint disease, pleural effusion and pericardial or peritoneal disease. The TB epidemic had not reached its peak in Africa before it was exacerbated by the advent of HIV infection (Hawken *et al.,* 1993, Harris, 1997).

1.1.3 The epidemiology of TB in The Gambia

The Gambia is a small coastal country in West Africa surrounded by Senegal. The Gambia lies between latitude 16° 34W and longitude 13 °28 N of the equator. Five ethnic groups predominate: Mandinka (42%), Wolof (16%), Fula (18%), Jola (10%), and Serahuleh (9%). Manjago, Serere, Bambara and Aku form the remaining 5%. Significant proportions (40%) of the population live in the West in or around the major towns of Serrekunda, Banjul and Brikama. There is intermarriage between these ethnic groups and

marriages are often transient. It is common place to find single parent families making family studies more difficult. The people are also highly mobile and it is a big endeavour to track down family members because they may be at opposite ends of the country.

The joint Leprosy and Tuberculosis Control Programme has been in place since 1986 and is run under a joint agreement between The Gambian Government and the Dutch agency Nederlandse Stichlting voor Lepraobeshtrijding. Prior to 1994, 94% of tuberculosis had not been treated (Lienhardt, *et al.,* 1998) and BCG was administered in sporadic vaccination campaigns of school children and among children and adults in the community. There are chest clinics in different parts of the country. Eighty-eight percent of treated cases were smear positive. Culture using specimens by both commercial Lowenstein Jensen slopes and BACTEC (Becton Dickinson) became available at the Medical Research Council Laboratories in Fajara in 1997. A retrospective review of patient notes indicated that 6.2% of tuberculosis patients were positive for HIV (Lienhardt *et al.,* 1998). Records for 1994 and 1995 show that 78% of smear positive patients were either cured or had completed treatment, 4.3% were treatment failures, and 4.6% had died, with mortality significantly higher among HIV patients (Lienhardt *et al.,* 1998).

The prevalence of drug resistance among *M. tuberculosis* strains in The Gambia is presumed to be minimal although this is currently under investigation. Statistics for The Gambia shows that there are one thousand new cases per year; for a country with a population of a little over a million the incidence is 1 case per thousand per year. TB is therefore a serious public health problem in The Gambia (WHO bulletin 2000).

1.1.4 The pathogenesis of TB

There are two types of pulmonary TB: primary and secondary TB. Following infection with *M. tuberculosis*, primary TB results from parasitisation and activation of alveolar

macrophages and M cells in mice (Hirsch *et al.,* 1994, Teitelbaum *et al.,* 1999). Upon inhalation of bacilli contaminated droplets, most of these particles settle in the upper respiratory epithelium and are expelled by the mucocilliary escalator. Only a few droplets reach the respiratory bronchioles and alveoli in the peripheral lung tissue. The bacilli contained within these droplets are then engulfed by alveolar macrophages, which are transformed into epithelioid cells (Schlesinger, 1996). Chemotactic factors attract circulating monocytes, lymphocytes and neutrophils leading to the formation of granulomatous focal lesions. This non-specific defence develops within a few days, limits the spread of infection and may achieve complete resolution. After about 3 weeks, antigen specific defences develop which contribute to the resolution of infection. After 4-5 weeks of progressive infection microscopic granuloma enlarge as individual foci expand and coalesce. This results in relatively large areas of necrotic tissue. *M. tuberculosis* bacilli are unable to multiply within this caseous tissue, due to its acid pH, low availability of oxygen and the presence of toxic fatty acid (Dannenberg and Rook, 1994). With intact cell mediated immunity (CMI) the infection may be arrested permanently at this point. However, *M. tuberculosis* is well adapted to its host and can survive for many years within an infected individual. The granuloma subsequently heals, leaving small fibrous and calcified lesions. If CMI is insufficient, haematogenous and lymphatic dissemination may occur. Enlarged hilar lymph nodes can rupture into adjacent airways, releasing necrotic material and causing tuberculous bronchopneumonia, while more distal spread results in miliary TB (Dannenberg and Rook, 1994).

Secondary (postprimary) TB occurs in two ways, either by reinfection or by reactivation of a dormant primary lesion. Until recently, it was generally believed that most post primary TB in developing nations was reactivation. However, DNA fingerprinting of the organisms involved, has shown that reinfection accounted for 75% of recurrent TB in a

South African study (van Rie *et al.,* 1999). In either case, a localised hypersensitivity reaction is the characteristic response, accompanied by tissue necrosis and caseation. Lymphocytes and other cells converge upon the site forming a wall of fibrous tissue to seal the necrotic site. There may be extensive tissue destruction resulting in the formation of cavities, and healing occurs by fibrosis rather than calcification (Dannenberg and Rook, 1994). An open cavitating lesion can leak infectious material directly into the bronchus, resulting in continuous discharge of bacilli into the sputum. In this instance, the individual is most infectious to others. Leaked bacilli can also be inhaled into other portions of the host lungs, resulting in tuberculous bronchopneumonia. If the growing granulomatous lesion erodes the wall of a vein, organisms can spread in via the circulation resulting in disseminated disease (Dannenberg and Rook, 1994).

The mechanisms that govern dormancy and reactivation, within either the organism or the host are not yet understood. Any condition that compromises cellular immunity makes reactivation more likely. However, the majority of individuals who develop tuberculosis have no obvious immunocompromise, suggesting host factors play an important role. It would appear that such individuals do not develop a truly protective immune response.

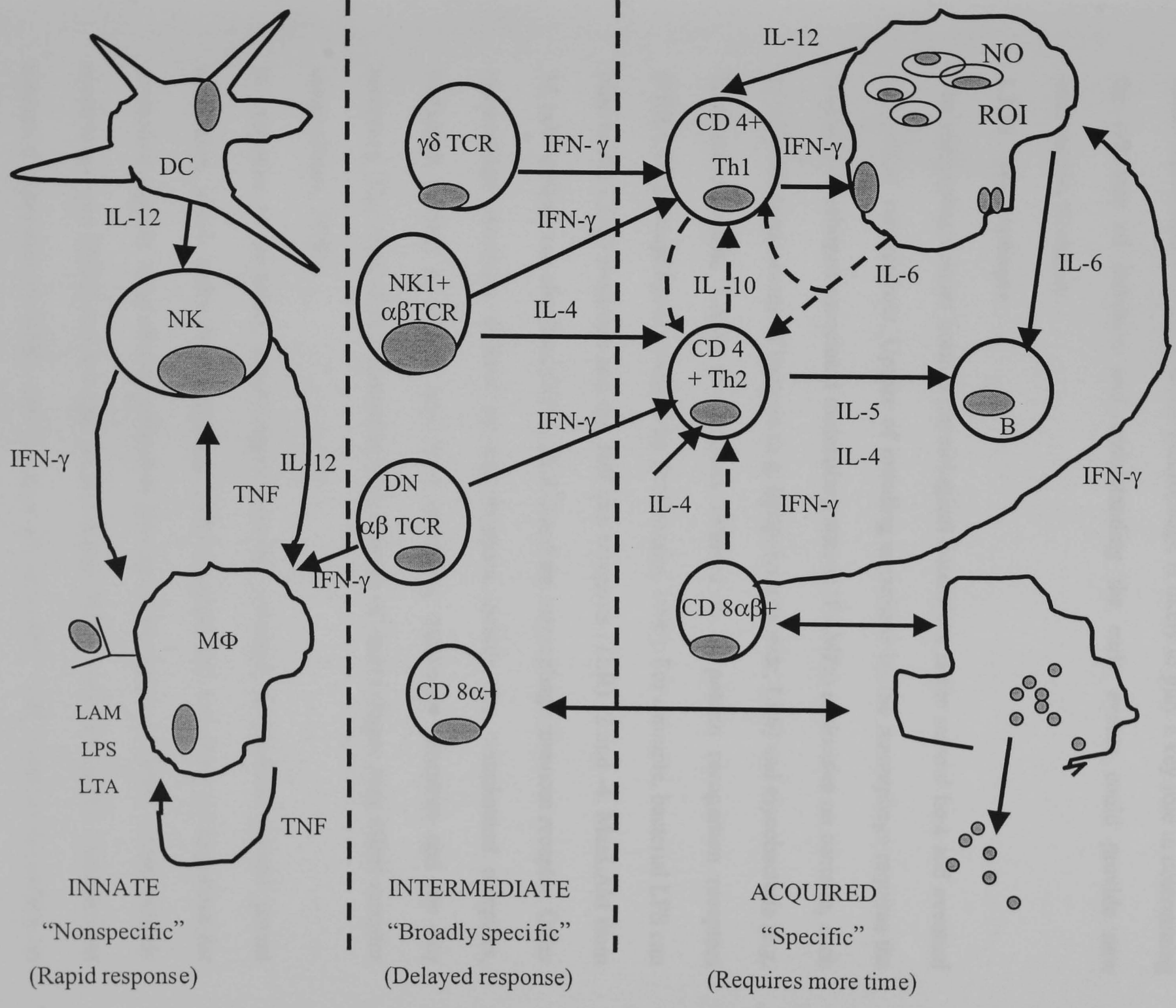
1.2 Immune responses to *M. tuberculosis* infection

M. tuberculosis is a facultative intracellular parasite that grows within mononuclear phagocytes. For this reason, the immune response to *M. tuberculosis* is predominantly cellular, requiring the co-ordinated activity of a wide range of T cell and macrophage functions. Much of our current understanding of mycobacterial immunity is derived from murine studies: the use of genetically engineered mice which lack specific components of the immune response has been particularly enlightening. However, *M. tuberculosis* is not a natural pathogen in mice and extrapolation of murine data to humans requires caution.

The immune system comprises two arms – innate immunity, which consists of non-specific mechanisms present prior to an encounter with a particular organism, and acquired immunity which, is specifically induced by foreign antigens. The two arms are bridged by antigen presenting cells such as dendritic cells, and CD1 cells and γδT cells. Figure 1.1 summarises the inter-relationship between the various pathways involved in the immune response to mycobacteria.

FIG. 1.1 Pathways of mycobacterial immunity.

Figure 1.1 overleaf shows the interaction between the innate and acquired immune system and the cells thought to bridge the gap. All abbreviations are listed in pages xiv and xv.


DC
IL-12
NK
IFN-γ
IL-12
TNF
MΦ
LAM
LPS
LTA
TNF
γδ TCR
IFN- γ
IFN-γ
NK1+
αβTCR
IL-4
DN
αβ TCR
IFN-γ
IFN-γ
CD 8α+
CD 4+
Th1
IFN-γ
IL-12
NO
ROI
IL-6
IL -10
CD 4
+ Th2
IL-5
IL-4
IL-4
IFN-γ
B
IFN-γ
CD 8αβ+
INNATE
"Nonspecific"
(Rapid response)
INTERMEDIATE
"Broadly specific"
(Delayed response)
ACQUIRED
"Specific"
(Requires more time)

1.2.1 Innate immunity

The macrophage is central to the innate immune response to mycobacterial infection. The initial interaction with the alveolar macrophage is likely to play a key role in determining the outcome of infection and understanding the early events could provide new intervention strategies.

1.2.1i Macrophages

The macrophage under normal physiological conditions is the natural host and eventual killer of *M. tuberculosis*. Uptake of invading organisms by the macrophage requires the binding of pathogen associated molecular patterns (PAMPs) molecules on bacteria, such as cell-wall components of bacteria (e.g. lipopolysaccahride, LPS) and mycobacteria (e.g. lipoarabinomannan, LAM) to receptors referred to as pattern recognition receptors (PRRs) on macrophages (reviewed by Schlesinger, 1996). For example, bacterial LPS can bind to the CD14 molecule and the Toll-like receptors (TLR) -2 and -4. ManLAM from *M. tuberculosis* can also bind CD14, TLR-2 and the macrophage mannose receptor. Other macrophage receptors utilised by mycobacteria include the complement receptors, surfactant protein A (Sp-A) and Sp-A receptors, scavenger receptors and the Fcγ receptors. The state of differentiation or activation of macrophages may affect receptor usage (Ernst, 1998).

In response to invasion, macrophages become activated to produce several potent molecules, which make the macrophage milieu hostile and therefore are important for protection against intracellular pathogens (reviewed by Murray, 1999). These include reactive oxygen (ROI) and nitrogen intermediates (RNI), toxic hydrolytic enzymes and increased expression of MHC class II and or the non-MHC CD1 molecule depending on the macrophage receptor utilised for uptake. There is rapid up regulation of the early gene KC, a neutrophil-specific chemoattractant belonging to the IL-8 related C-X-C family of

small peptide cytokines, production of cytokines such as tumour necrosis factor (TNF), interleukin 1β (IL-1β), interleukin-2 (IL-2), IL-8 and IL-10. Activation of macrophages also results in up-regulation of natural resistance associated macrophage protein 1 (NRAMP1) and nitric oxide synthase, the enzyme that catalyses the production of the free radical nitric oxide (NO). The function of NRAMP1 is discussed in more detail in section 1.3.2ia. The above macrophage functions are greatly enhanced by the cytokine interferon-γ (IFN-γ), produced by T cells and natural killer cells (Nathan *et al.,* 1983).

Despite the production of such potent molecules, it is known that viable *M. tuberculosis* can use several mechanisms to evade killing by macrophages. One such mechanism is to inhibit fusion of early endosomes with acidic late endosomes and lysosomes thereby disrupting antigen presentation to T cells. This does not occur in the presence of a functional NRAMP1 protein (Hackam *et al.,* 1998).

Murine peritoneal macrophages can be readily activated with IFN-γ to inhibit virulent *M. tuberculosis* growth (Rook *et al.,* 1987). Ironically, normal human monocytes in the absence or presence of any activating stimulus are less able to inhibit *M. tuberculosis* growth than murine macrophages. Even the effect of 1, 25-dihydroxyvitamin D, known to exert a slightly greater effect than other stimuli in mice, induces less inhibition of *M tuberculosis* in human macrophages than is readily achieved by incubating murine macrophages in IFN-γ (Rook *et al.,* 1987). Other pathways may be more important in humans. Lammas *et al.,* (1997) reported that exogenous ATP mediates lysis of *M. bovis* BCG-infected human macrophages via P2Z receptors, resulting in death of mycobacteria.

1.2.1ii Natural killer (NK) Cells

Natural killer cells are a subset of bone marrow derived leukocytes with a granular morphology, originally characterised by their ability to kill tumour cells in a non- MHC restricted manner. They do not express αβ or γδ T cell receptors or surface

immunoglobulins and can develop in the absence of functional T and B-lymphocytes. NK cells are involved the in early response to intracellular infections (Trincheri, 1989). They are capable of producing IFN-γ in response to the macrophage cytokines IL-12 and TNF-α, induced by mycobacterial antigens (Bancroft *et al.,* 1993). Reports have suggested three mechanisms by which NK cells can contribute to host resistance. Firstly, they can bind directly to pathogens and in some cases achieve killing of the organisms directly. Secondly, they can mediate cytotoxic activity against host cells infected with intracellular pathogens. Finally; they can be triggered to secrete a range of cytokines including IFN-γ and involved indirectly in resistance of the host. It is possible that alveolar macrophages ingest mycobacteria and produce IL-12 thereby favouring the development of T helper 1 type responses (see below) via the induction of IFN-γ production and enhancement of the cytotoxicity of NK cells.

1.2.1iii Dendritic cells (DC)

Dendritic cells (DC) are antigen presenting cells (APC) that have been shown to be powerful tools for manipulating the immunologic function of B and T lymphocytes, the main mediators of acquired immunity (Banchereau and Steinman, 1998). They function by capturing antigens, process them and on activation express lymphocyte co-stimulatory molecules. They migrate to lymphoid organs and secrete cytokines to initiate immune responses. They present processed antigens to antigen specific T cells, thereby exhibiting an essential role in the initiation of primary T cell responses to foreign antigens. Initially, the precise role of DCs in the uptake of *M. tuberculosis* was not clear. An earlier study reported that blood DCs were not detectably phagocytic for mycobacteria and further studies failed to show any evidence for the transfer of mycobacterial antigens from macrophages to dendritic cells in a form that was stimulatory for T cells (Pancholi *et al.,*

1993). However, recent studies demonstrate that DCs generated from human peripheral blood by short term culture in medium containing recombinant human granulocyte-macrophage-colony stimulating factor and IL-4 were capable of phagocytosing *M. tuberculosis*. Also, bacilli discharged by engulfing epithelial M cells are taken up by DCs and macrophages associated with the T cells of the parafolicular area (Lugton, 1999). It is now clear that apart from activation and maturation of DCs, DCs pulsed with bacilli have been used to prime antigen specific $CD8^+$T cells for lysis (Mohagheghpour *et al.,* 1998). Infection of DCs with live *M. tuberculosis* bacilli resulted in increased APC surface expression of the co-stimulatory molecules CD40 and B7.1 as well as MHC class I molecules. In addition, infected DC secrete elevated levels of inflammatory cytokines including TNF-α, IL-1 and IL-12 (Henderson *et al.,* 1997). Activation of DCs may facilitate their migration to lymph nodes and enhance presentation of antigen to T cells thereby facilitating the induction of the immune response against *M. tuberculosis.*

1.2.1iv Gamma delta T cells (γδT cells)

Gamma delta (γδ) T cell receptor (TCR) bearing T cells have been identified in murine and human TB lesions. Early responses to mycobacterial antigens in naïve individuals have been studied in new-borns, where γδT cells were shown to expand in response to *M. tuberculosis* (Boom *et al.,* 1994). Activated γδT cells can secrete cytokines such as TNF-α, IL-4, IL-5, IL-10, IL-2, IFNγ and also exhibit cytolytic activity against macrophage targets pulsed with *M. tuberculosis* (Munk *et al.,* 1990; Follows *et al.,* 1992). The protective role of these cells is less certain as they have not been detected in materials from tuberculous pleural effusions or tuberculous lymph nodes (Tazi *et al.,* 1992; Barnes and Modlin, 1996). They have been shown to recognise phoshorylated *M. tuberculosis* antigens (Lang *et al.,* 1995) and a 5' triphosphorylated thymidine compound from *M. tuberculosis* (Constant *et al.,* 1994). Expansion of *M. tuberculosis* γδT cells is greater in

healthy tuberculin reactors and in patients with tuberculous pleuritis than in those with advanced pulmonary and miliary tuberculosis, suggesting that γδT cells may contribute to immune resistance (Barnes *et al.,* 1992).

1.2.2 Presentation of mycobacterial antigen to T cells.

Mycobacterial antigens are presented to T cells by different mechanisms depending on the nature and source of antigen been presented. The major histocompatibility complex (MHC) restricted pathway involving protein antigens is the classical method. There is also a recently unravelled non-MHC restricted pathway utilising CD1b molecules and these present non- protein antigens to either double negative IFN-γ producing T or $CD8^+$ T cells (Lewinshon *et al.,* 1998).

1.2.2i Human leukocyte antigens (HLA)

In human, the MHC is referred to as human leukocyte antigens (HLA), is encoded on the short arm of chromosome six and is very polymorphic (reviewed by Klein and Sato, 2000). The HLA restricted pathway consists of four distinct types of molecules, only two are involved in antigen presentation: the class I and class II molecules which classically recognise peptides in context of either $CD8^+$ or $CD4^+$ T cells respectively. They are cell surface glycoproteins receptors that bind peptide and present them to T cells. Virtually, all cells of the body express class I and present endogenous antigens whereas, class II molecules are expressed by a limited number of host cells; these are B cells, dendritic cells and macrophages. Unlike the innate receptors that recognise chemical patterns shared by groups of bacteria, the HLA molecules recognise structural specificity, in that the antigen has to be processed and refined into small peptides for presentation to T cells. The structure of these HLA molecules determine how well they will bind to peptides and present them to T cells. Susceptibility or resistance to disease has been related to binding

capacity of these molecules to peptides. A number of associations between HLA alleles and several diseases have been reported mainly with autoimmune diseases. There are many reports of HLA associations with TB. Some have demonstrated positive associations (Brahmajothi *et al.,* 1991, Bothamley *et al.,* 1989) some no association (Hawkins *et al.,* 1988, Cox *et al.,* 1982) and others negative associations (Singh *et al.,* 1983).

1.2.2ii Cluster differentiation 1b (CD1b) Molecule

Non- peptide antigens such as mycolic acids, lipoarabinomannan related phosphoinositol mannosides and glucose mycolates utilise the CD1 pathway by stimulating either $CD8^+$ or $CD8^-$, $CD4^-$ double negative and or IFN-γ producing T cells (Brenner *et al.,* 1998). These cells are efficient cytolytic T cells capable of lysing infected target cells. Whether CD1 associated glycolipid antigens derived from mycobacteria can elicit protective host response is under investigation. Studies of human leprosy have provided direct evidence for the involvement of CD1 restricted T cells in host responses to infection. CD1a, b and c are expressed on dendritic cells in granulomata within the skin lesions of leprosy patients (Sieling *et al.,* 1998). Furthermore, the frequency of $CD1^+$ cells correlates with levels of cell mediated immunity to *M. leprae*, being more abundant in the granulomata of patients with the immunologically responsive tuberculoid form of the disease when compared with the unresponsive lepromatous form (Sieling *et al.,* 1998). Also, IL-10 found specifically at the site of infection in the lepromatous form has been shown to down regulate CD1 expression (Sieling *et al.,* 1998). More recently, *M. tuberculosis* reactive CD1 restricted T cell lines from peripheral blood of patients with tuberculosis have been derived. The study of CD1 restricted T cells from patients with mycobacterial infection as well as normal healthy donors showed that all CD1 restricted T cell lines

isolated produced high levels of IFN-γ but little or no IL-4 upon stimulation with mycobacterial antigens (Sieling *et al.,* 1998).

1.2.3 Acquired Immunity

There are two main types of lymphocytes (B and T cells). The presence of distinct glycoproteins known as clusters of differentiation (CD) antigens on surface of T cells and other cells of the immune system serve as phenotypic markers allowing distinction between populations or subsets with different functions in the immune system. For example, CD19 and CD22 are expressed on all B cells and CD2 and CD3 on all T lymphocytes. The B cells are involved in humoral responses and are thought to have no major role in the containment of TB. One the other hand, the T cells are involved in CMI and can be differentiated into two main subtypes; $CD4^+$T and $CD8^+$T cells. $CD4^+$ cells are described as "helper" cells which release cytokines that attract phagocytes to the site of infection, and those expressing $CD8^+$ are said to belong to the "suppressor", "killer" or "cytotoxic" subset which kill infected macrophages (Reinherz and Schlossman, 1980). Others T cell subsets include the CD1 restricted $CD4^-CD8^-$ double negative T cells, and γδT cells.

1.2.3i Cluster differentiation 4 ($CD4^+$) T cells

Lymphocyte sub-population expressing CD4 have been shown experimentally and clinically to have a dominant but not exclusive role in immune defences against TB. Mice depleted of $CD4^+$T cells prior to infection with *M. bovis* are unable to control mycobacterial growth, whereas depletion of $CD8^+$T cells shows variable effects (Pedrazzini *et al.,* 1987). In humans, $CD4^+$T cells are selectively expanded at the site of disease in patients with a protective immune response, for example those with

tuberculous pleuritis (Barnes *et al.,* 1989). Depletion of $CD4^+$ T cells by HIV infection markedly increases susceptibility to primary and reactivation TB.

$CD4^+$T cells can be further subdivided into two functionally distinct groups: T helper type1 (Th1) and T helper type 2 (Th2) cells based on their cytokine production profile (Mosmann *et al.,* 1986). Th1 cells produce interleukin 2 (IL-2) and IFN-γ and execute cell-mediated immune response such as delayed hypersensitivity and macrophage activation. Th2 cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 and assist in antibody production for humoral immunity. It is possible that the dominant type of antigen specific $CD4^+$ T cell clone present at the site of infection determines the outcome of disease. Distinct microbial antigens may favour dominance of Th1 or Th2 cells. For instance, in leprosy patients, Th1 cytokines (IFN-γ and IL-2) predominate in the skin lesions of tuberculoid leprosy patients who mount a resistant immune response to *M. leprae,* whereas Th 2 cytokines (IL-4 and IL-10) are prominent in lepromatous leprosy lesions with ineffective immunity and enormous bacillary burdens (Seiling and Modlin, 1994). Published data on cytokine production by human T cells in response to *M. tuberculosis* are conflicting. Many studies indicate that most $CD4^+$T *M. tuberculosis* reactive T cell clones propagated in vitro are Th1 like, producing high concentrations of IFN-γ but low concentrations of IL-4 and IL-5 (Del Prete *et al.,* 1991). Boom *et al.,* (1991) demonstrated *M. tuberculosis* reactive T cell clones capable of secreting IFN-γ and IL-4. Barnes *et al.,* (1993) indicated that most *M. tuberculosis* reactive clones produce both Th1 and Th 2 cytokines including IFN-γ, IL-2, IL-5 and IL-10. However, mRNA expression of Th1 cytokines such as IFN-γ and IL-2 in patients with tuberculous pleuritis is greater in pleural fluid mononuclear cells than in blood mononuclear cells and concentrations of IFN-γ are higher than those in serum (Barnes *et al.,* 1993). Pleural fluid lymphocytes stimulated with *M. tuberculosis* produce more IFN-γ and more IL-2 than do peripheral

blood lymphocytes. These results provide strong evidence for selective concentration of Th1 cells at the site of disease in persons with a protective immune response and suggest that Th1 cells play an important role in human antimycobacterial defences. IFN-γ in combination with TNF enhances the antimycobacterial activity of murine macrophages, probably through increased production of reactive nitric oxide metabolites (Flesch and Kaufmann, 1991). IFN-γ also stimulates human macrophages to produce TNF which facilitate mycobacterial elimination (Rook *et al.,* 1987). IL-2 causes T cell division further increasing the local concentration of macrophage activating factors secreted by T cells. In contrast to the effects of Th1 cytokines, IL-4 deactivates macrophages and blocks T cell proliferation by down regulation of IL-2 receptor expression (Martinez *et al.,* 1990) and may therefore inhibit the immune response to *M. tuberculosis*.

Although $CD4^+$T cells are traditionally known as cytokine producers, they are also capable of cytotoxic activity. $CD4^+$ cytotoxic lymphocyte (CTL) clones specific for purified protein derivative (PPD) effectively lyse a variety of targets using both the Fas-Fas ligand (FasL) pathway and the granule exocytosis pathway of cytotoxicity (Oddo *et al.,* 1998). Another study showed lysis of monocytes infected with *M. tuberculosis* by recombinant FasL resulting in the actual killing of intracellular bacteria (Lewinsohn *et al.,* 1998).

1.2.3ii Cluster differentiation 8 ($CD\ 8^+$) T cells.

Kaufmann described a potential role of $CD8^+$T cells in protective immunity against TB in 1988. Several studies using $\beta_2$microglobulin (β_2 M) knock out mice have been used to illustrate the importance of $CD8^+$T cells in mycobacterial infections (Flynn *et al.,* 1992; Ladel *et al.,* 1995). One such study demonstrated that disease dissemination to the spleen is enhanced in β_2M knock out mice compared to controls (D'Souza *et al.,* 2000). Other

studies using transporters associated with antigen presentation (TAP) knockout mice showed that $CD8^+$ CTLs that are MHC class 1 restricted may present secreted mycobacterial antigens through a mechanism that involves the TAP transporter system (Mazzaccaro *et al.,* 1996, Behar *et al.,* 1999).

Thus far, evidence in favour of a protective role for MHC class I restricted CTLs in suppression of *M. tuberculosis* is emerging. In mice, cytolytic $CD8^+$T cells are able to recognise and kill culture filtrate pulsed macrophages using the secretion of perforins as the main mechanism of cytotoxicity. In humans, small peptide such as ESAT6 specific CTLs were used to demonstrate a non-lytic mechanism where T cell cytokines activate macrophages to induce factors that accumulate to suppressive levels after 6 days of culture (Lalvani *et al.,* 1998). There is evidence that a defined epitope of the 19KD lipoprotein of *M tuberculosis* elicits MHC class I restricted CTLs with the ability to lyse autologous monocytes infected with avirulent mycobacteria (Mohagheghpour *et al.,* 1998). Lewinsohn *et al.,* (1998) identified $CD8^+$CTLs that killed *M. tuberculosis* infected dendritic cells. A non-polymorphic MHC class 1b pathway was proposed rather than a MHC class 1 presentation pathway (Lewinsohn *et al.,* 1998). Work in The Gambia has demonstrated a role for $CD8^+$T cells in mycobacterial immunity in response to both BCG and *M. tuberculosis* (Turner and Dockrell, 1996; Turner *et al.,* 2000).

1.3 Genetic susceptibility to mycobacterial disease

1.3.1 Evidence that host genetic factors influence susceptibility to tuberculosis

In the nineteenth century, when the incidence of TB reached epidemic proportions, an inherited susceptibility to tuberculosis was suspected based on the observation that the incidence of TB in relatives of affected individuals appeared to be increased. Although this concept lost credibility when Koch discovered that TB was caused by infection with *M. tuberculosis*, a number of observations over the years rekindled interest in the genetic component of mycobacterial susceptibility.

The majority of individuals infected with *M. tuberculosis* do not get disease: only 10% of infected persons will develop clinical symptoms during their life (Murray *et al.,* 1990). Convincing epidemiological evidence to suggest that host genetic factors play an important role in determining susceptibility or resistance to mycobacterial infection comes from an incident in Germany, prior to the development of antimycobacterial chemotherapy. In 1926 two hundred and fifty-one children from Lubeck were accidentally immunised with a virulent strain of *M. tuberculosis* (Smith, 1988). Of these, 47 (20%) developed no detectable disease, 127 (50%) developed radiologically evident disease and 77 (30%) died. This suggests that the ability of the organism to cause disease varies between individuals and this variability may largely be attributed to differences in the host's ability to resist disease. Geographically remote populations such as Eskimos in the North Pole, where *M. tuberculosis,* is not endemic are particularly susceptible and develop rapidly progressive, often-fatal, disease. Black people are twice as likely to develop clinical disease than whites (Stead, 1992). Considerable evidence of a role for genetic factors in the clinical outcome of mycobacterial infections comes from twin studies (Kallman and Reisner, 1943). Concordance rates for tuberculosis are higher

among monozygotic twins when compared to dizygotic twins, siblings or marriage partners.

The genetics of host resistance or susceptibility to mycobacteria is still poorly understood. In terms of Darwinian natural selection, the development of host resistance to a given parasite requires that it produce a high mortality for its host before the reproductive period. In this way only the more resistant survivors reproduce. Over many generations of continued selective pressure from the parasite, the percentage of resistant individuals gradually increases. Malaria is a good example of the selective pressure infection might have on human evolution – polymorphisms in at least 12 genes confer resistance to malaria, the sickle mutation in the beta-globin gene being the best known (Weatherall, 1996). Since a quarter of all deaths at the time were due to TB, it is possible that the epidemic in Europe in the 16th to 19th centuries waned as a result of natural selection, though other factors such as the development of isolation facilities were probably equally important. In support of this, genetic and epidemiological studies have provided some evidence that the high frequency of Tay-Sachs disease in certain Jewish populations reflects heterozygous resistance to tuberculosis in some of the ghettos of Eastern Europe (O'Brien, 1991).

1.3.2 **Approaches for identifying TB susceptibility genes.**

At least four approaches can be used to identify disease susceptibility genes in complex diseases such as TB. Firstly, animal models have obvious logistical advantages, but the question remains as to how reliably data from mice extrapolates to man. Secondly, a number of rare but informative single gene defects in human families have led to the identification of putative mycobacteria susceptibility genes in man. Thirdly, a complex phenotype may be viewed as a constellation of sub-phenotypes (quantitative traits), which

in turn are regulated by their own sets of genes. Thus by simplifying the phenotype, fewer genes are involved, making them easier to identify. Finally, developments in complex trait genetic analysis such as semi-automated genotyping of highly polymorphic microsatellites dispersed throughout the genome, and the statistical packages to analyse the data generated has allowed both linkage and association study designs using candidates gene and genome wide scanning approaches in TB in outbred human populations.

1.3.2.i Animal models

Animal models offer the opportunity to study hundreds of meioses from a single set of parents. The use of animal models is enhanced by the relative ease of genetic manipulation, for instance the production of 'knock-out' animals. Animals can be reared in the same conditions, reducing variation due to environmental factors. A potential disadvantage is that the genes shown to be important in inbred strains of mice could differ from those relevant in humans, but findings could still be helpful for identifying biochemically similar pathways in humans. Animal studies using selective breeding techniques were used to identify two distinct phenotypes of disease after inhaling large numbers of *M. bovis* (Lurie, 1952). Highly resistant rabbits had localised chronic pulmonary lesions like those found in immunocompetent adult humans with TB, whereas susceptible rabbits showed rapidly progressive generalised spread of infection, even with smaller numbers of *M. bovis,* similar to that found in infants and immunocompromised people. Although not the best model for human TB, developments in immunological and genetic technology (e.g. genetically engineered 'knockout' animals and the availability of monoclonal antibodies) have favoured the mouse model and a number of candidate TB susceptibility genes have subsequently been identified.

1.3.2.i.a Natural resistance associated macrophage protein 1 (*Nramp1*)

Nramp1, a gene originally identified almost 30 years ago as *Ity/Lsh/Bcg* for its role in controlling *Salmonella typhimurium, Leishmania donovani* and *M. bovis* infection in mice, was the first murine mycobacteria susceptibility gene to be identified. It regulates a cascade of gene induction events mediating inflammation, elimination of invading organisms and induction of T-cell memory against re-infection (Blackwell, 1989). Following the mapping of *Lsh* ands *Ity* loci to murine chromosome 1 (Plant *et al.,* 1982), Gros *et al.,* (1981) showed that resistance to infection with *M. bovis* (BCG Montreal) was under the control of a single gene *Bcg* which mapped to the same region of chromosome 1 in mice. The gene was positionally cloned and renamed *Nramp* 1 (Natural resistance associated macrophage protein, Vidal *et al.,* 1993). A non-conservative amino acid substitution of glycine by aspartic acid at position 169 was shown to be responsible for the susceptible Bcg^s phenotype in 27 inbred mouse strains (Vidal *et al.,* 1993; Malo *et al.,* 1995).

Nramp1 protein localises to the late endosomal compartment in resting macrophages and after phagocytosis is recruited to the membrane of the phagosome (Searle *et al.,* 1998). There is evidence that it may restrict the replication of intracellular pathogens by altering the phagolysosomal environment. Functional studies using mice and transfected macrophage lines have shown that Nramp1 may be important early in macrophage activation. It has many pleiotropic effects on macrophage function including regulation of the expression of the chemokine KC, IL-1β, inducible nitric oxide synthase (iNOS), MHC class II molecules, and TNF. It also modulates nitric oxide (NO) release, L-arginine flux, the oxidative respiratory burst, and tumoricidal and antimicrobial activity (Blackwell, 1989, Skamene, 1994, Radzioch *et al.,* 1995). Macrophages carrying the susceptible *Nramp1* allele have been shown to have a defect in the ability to process

antigens (Lang *et al.,* 1997), which is compounded by the influence of the gene on molecules regulating (TNF, IL-1β) or directly involved in (MHC class II) antigen presentation. This results in an in vivo bias towards the development of a Th 1 type response in mice bearing the wild-type infection resistant allele, while a Th 2 type response is elicited in susceptible mice (Kaye and Blackwell, 1989; Kramnick *et al.,* 1994). However, a study of *Nramp1* deletion and *M. tuberculosis* infection in 129vs mice failed to show any difference in bacterial growth in major organs and survival times in mice infected with the virulent H37Rv strain of *M. tuberculosis* (North *et al.,* 1999). In addition, an earlier study failed to show any influence of *Nramp*1 on the growth of *M. tuberculosis* and a further mouse strain identified as being *Bcg*^{r} were shown to be more susceptible to this infection. The inference that *Nramp1* mutant mice are susceptible to infection with all mycobacterial species may be incorrect for *M. tuberculosis* (Medina and North 1996, North 1999).

1.3.2.i.b Other candidate genes in mice

Experiments with 'knock out' mice have been used to define the role of many other genes in TB. For example, it has been shown that mice with targeted disruption of the gene encoding IFN-γ (*Ifnγ* $^{o/o}$) or the IFN-γ receptor1 gene (*Ifnγr1* $^{o/o}$) are markedly more susceptible to infection with *M. bovis* BCG (Kamijo *et al.,* 1996) or with virulent *M. tuberculosis* (Cooper *et al.,* 1993). Mice lacking *Ifnγr1* showed a decrease in hepatic granuloma formation at 2 weeks after inoculation, decreased MHC class II antigen expression, and significantly reduced production of TNF after *M. bovis* BCG infection or LPS challenge. Macrophages from mice lacking *Ifn*-γ were unable to produce nitric oxide when compared to macrophages from wild type mice. In wild type mice TNF was shown to be essential for the recovery from *M. bovis* BCG infection and for mounting of a protective immune response against *M. tuberculosis*. Thus IFN-γ acting in synergy with

TNF is apparently essential for resistance to mycobacterial infection and their major role is thought to be the activation of macrophages for monokine production leading to activation of other pathways required for the destruction of mycobacteria.

Other genes which, when disrupted in mice, lead to increased susceptibility to mycobacterial infection are summarised in table 1.1 below. As more such models are developed, a clearer picture should emerge as to the role of individual genes in protection against mycobacterial infection and the ways in which these genes interact.

Table 1.1. Effect of disruption of various candidate genes on susceptibility to mycobacterial infection

Gene	Effect*	Reference
TNF- Receptor (55Kd)	*M. bovis* (BCG)	Flynn *et al.*, 1994
Interferon –γ	*M. tuberculosis*	Cooper *et al.*, 1993
		Flynn *et al.*, 1993
Interferon –γ receptor	*M. bovis* (BCG)	Kamijo *et al.*, 1993
β2-microglobulin	*M. tuberculosis M. bovis* (BCG)	Flynn et al ., 1992, Ladel *et al.*, 1995
MHC Class II	*M. bovis* (BCG)	Ladel *et al.*,1995a
T cell receptor	*M. bovis* (BCG)	Ladel *et al.*, 1995b
Recombinase activating gene	*M. bovis* (BCG)	Ladel *et al.*, 1995c
Interferon regulatory factor1	*M. bovis* (BCG)	Kamijo *et al.*, 1994
Inducible nitric oxide synthase	*M. tuberculosis*	MacMicking *et al.*, 1997
Interleukin –12	*M. tuberculosis*	Cooper *et al.*, 1997
Transporter associated with antigen processing1	*M. tuberculosis*	Behar *et al.*, 1998

* increased susceptibility to the organism listed

1.3.2ii Mendelian susceptibility to mycobacterial infection in man

A number of families have been identified in which there is an increased susceptibility to mycobacterial infection, which appears to be inherited as a single gene Mendelian

disorder (Engbaek, 1964, Uchiyama *et al.,* 1981, Levin *et al.,* 1995). These patients have selective susceptibility to poorly pathogenic mycobacteria such as *M. bovis* BCG and non-tuberculous mycobacteria (NTM). With the exception of other intracellular pathogens such as salmonella, individuals with idiopathic BCG and NTM infections do not generally have associated infections suggesting that a highly specific immune mechanism controls TB. Although rare, such families provide important insights as to the role of a specific gene in the immune response to mycobacteria.

The first recorded case of idiopathic BCG syndrome was probably in 1951 (Mimouni, 1951). In a retrospective study of all cases of disseminated BCG infection following routine vaccination in France, an underlying immunodeficiency was identified in less than half of the cases (Cassanova *et al.,* 1996). The prevalence in France is estimated at 0.59 cases per million births and the disease is not confined to any particular ethnic group or geographic region (Casanova, 1996). A Mendelian disorder of autosomal recessive inheritance was initially suggested by the high rates of affected siblings and parental consanguinity together with equal number of male and female patients. To date, mutations in four genes have been associated with Mendelian susceptibility to mycobacterial infection in man (Mendelian Inheritance in Man MIM 209950).

Figure 1.2 on page 28 illustrates the interaction between IL-12 and IFN-γ in mycobacterial immunity.

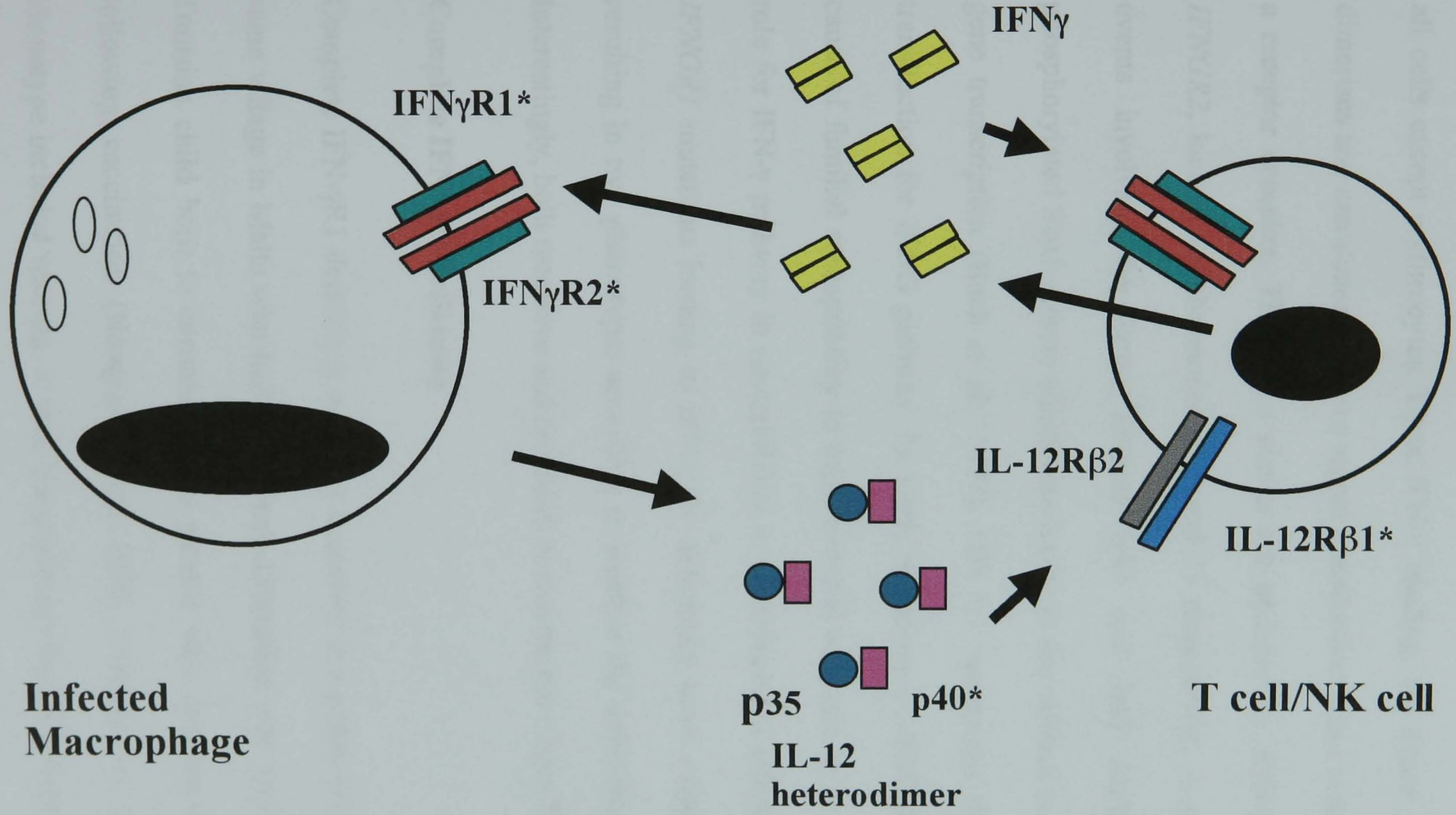


FIG.1.2 Mycobacterial immunity - the role of IL-12 and IFN-γ

Infected macrophages become activated and release the heterodimeric IL-12 cytokine which binds to its dimeric receptor on either NK cells or T cells. Binding of IL-12 to its receptor activates T cells to produce IFNγ. IFNγ released by NK cells and T cells binds to its dimeric receptor which is found on all cell types except erythrocytes. Mutations leading to increased susceptibility to mycobacterial infection have been identified in four genes in this pathway (*), highlighting the importance of IL-12 and IFN-γ in antimycobacterial immunity in man.

1.3.2ii a Interferon-γ receptor ligand binding chain (*IFNGR1*)

IFN-γ exerts its pleiotropic effects via a species-specific receptor, which is expressed on all cells except erythrocytes. Upon IFN-γ binding, the ligand binding chain (IFN-γR1) dimerises and associates with two signal transduction chain molecules (IFN-γR2) to form a receptor complex. These two chains are encoded by different genes, *IFNGR1* and *IFNGR2*, located on chromosomes 6 and 21 respectively. A series of phosphorylation events involving the Janus kinases Jak1 and Jak2 leads to the formation of phosphorylated Stat1 dimers which translocate to the nucleus to activate IFN-γ-inducible gene transcription (Bach *et al.,* 1997). FIG 1.3 summaries the mechanisms of signal transduction for IFNG pathway. In 1996, mutations in *IFNGR1* were identified as the cause of familial susceptibility to mycobacterial infection in two families establishing a role for IFN-γ pathway in susceptibility to mycobacterial infections. A number of other *IFNGR1* mutations leading to IFN-γ R1 deficiency have subsequently been identified resulting in two phenotypes according to whether the deficiency is partial or complete. Interestingly, both recessive and dominant mutations have been identified.

Complete IFN-γR1 deficiency

Complete IFN-γR1 deficiency was first described in a group of related children from the same village in Malta who had disseminated infection with various NTM species, and a Tunisian child born to consanguineous parents who developed disseminated BCGosis following vaccination (Newport *et al.,* 1996, Jouanguy *et al.,* 1996). The clinical phenotype included wasting, fevers, lymphadenopathy (histology showed multi-bacillary, poorly-differentiated lesions) and anaemia (Levin *et al.,* 1995).

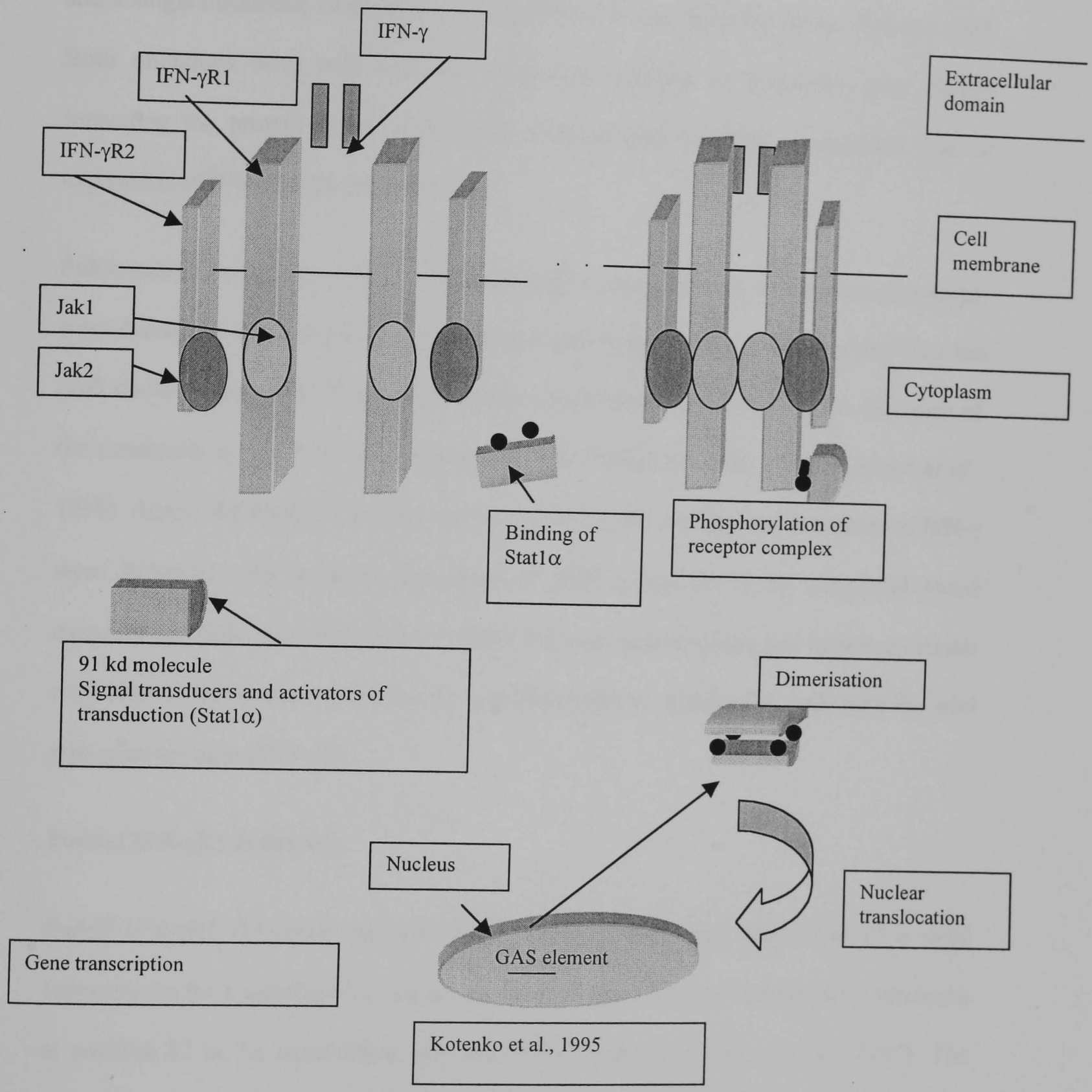


FIG.1. 3 Schematic drawing of mechanisms of signal transduction for IFN-γ pathway

Upon IFN-γ treatment, four components (IFN-γR1, Jak1, Jak2 and Stat1α) are phosphorylated rapidly at tyrosine residues. The receptor chains associate and this is thought to occur via Jak2 which may have a binding site on IFN-γR2 or Jak1. Stat1α dimerises and translocates to the nucleus and binds to the gamma activated site of any IFN-γ inducible gene.

A transversion of C to A at nucleotide position 395 was identified in the Maltese kindred, and a single nucleotide deletion of C at position 131 was identified in the Tunisian child. Both mutations were null recessive mutations resulting in premature stop codons, truncating the protein in the extracellular domain and resulting in complete lack of expression of IFN-γR1 on the cell surface.

Subsequently, a number of other mutations in this gene, resulting in the same phenotype, were described. One patient was a compound heterozygote who inherited a 4bp insertion (107 ins 4) within *IFNGR1* exon 2 from her father and a substitution of the first base of the consensus splice donor site in intron 3 (200 +1GA) from her mother (Altare *et al.*, 1998). Again, the receptor was not expressed on the cell surface, and responses to IFN-γ were defective with reduced production of TNF-α and IL-12 by peripheral blood mononuclear cells. A causative relationship between mutant alleles and impaired cellular responses to IFN-γ was established by complementation of defective cells with the wild type gene encoding IFN-γR1.

Partial IFN-γR1 deficiency

Partial IFN-γR1 deficiency has also been observed. The first report was of a child homozygous for a mutation causing an amino acid substitution of isoleucine by threonine at position 87 in the extracellular domain of the receptor (Jouanguy *et al.*, 1997). The receptor was expressed at the cell surface but the mutation reduces the affinity of the receptor for its ligand. This resulted in an intermediate phenotype, both clinically and in vitro. Whilst in vitro cellular responses from children with complete IFN-γR1 deficiency are abrogated, cells from children with partial deficiency responded to high IFN-γ concentrations, when compared to normal controls. The outcome of treatment with IFN-γ was favourable, and the patients are healthy and alive without on going therapy. It was

suggested that signalling mediated by IFN-γR, though impaired, was sufficient to promote morphologically mature granuloma formation.

Partial IFN-γR1 deficiency also results from autosomal dominant mutations described in 3 families and 9 sporadic cases with disseminated mycobacterial infection. All cases had a 4bp deletion, at nucleotide position 818bp of the 3' region the *IFNGR1*, thereby identifying a mutational hotspot within *IFNGR1* (Jouanguy *et al.*, 1999). The truncated protein lacks the intracellular signalling domain but is expressed at the cell surface and exerts a dominant–negative effect through impaired recycling, abrogated signalling but normal binding to IFN-γ. Patients developed disseminated infection from poorly virulent mycobacteria and *M. bovis* BCG.

Mutations identified to date in *IFNGR1* are summarised in figure 1.4.

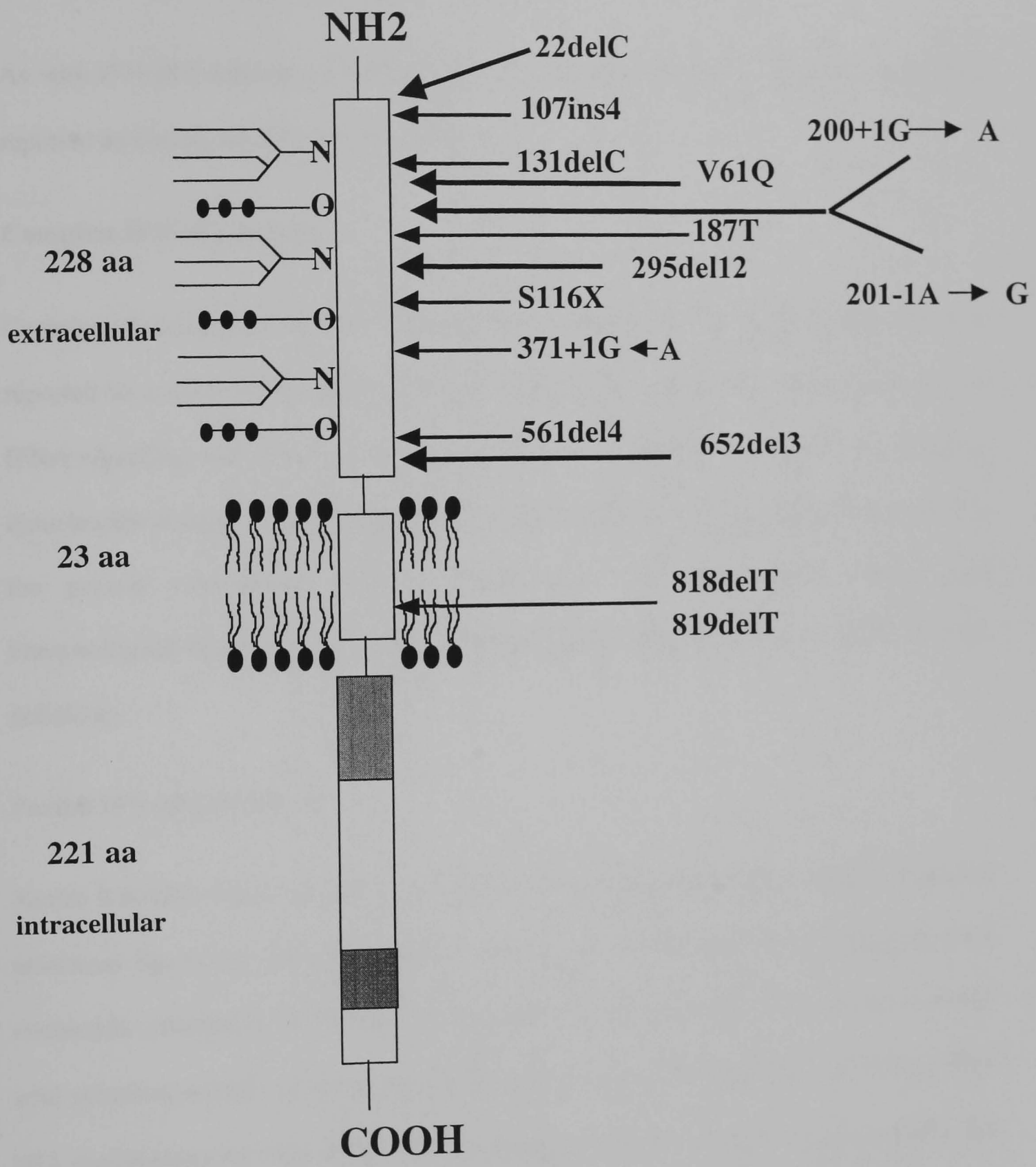


FIG.1.4. Model of IFNγR1

Model of amino acid structure of the IFNγR1 showing the mutations and deletion hotspots. The gray shaded areas of the intracellular domain are biological active regions which must be tyrosine phosphorylated for binding of the Jak1 (upper)and Stat1(lower) molecules. The extracellular domain consists of 228 amino acids, the transmembrane domain contains 23 amino acid and the intracelluar domain contains 221 amino acids. Most of the mutations are in the extracellular domain of the gene. The deletion hotspot at nucleotide position 818 is in the transmenbrane domain of the molecule. A 13 th missense mutation C77Y substitution of cysteine at nucleotide position 182.

1.3.2iib Interferon-γ receptor signal transduction chain (*IFNGR2*)

As with IFN-γR1 deficiency, both partial and complete deficiency of IFN-γR2 has been reported as a result of mutation in *IFNGR2*.

Complete IFN-γR2 deficiency

Complete deficiency of the IFN-γ receptor signal transduction chain (IFN-γR2) has been reported in a child with disseminated *M. fortuitum* and *M. avium* complex infections. IFN-γ signalling was absent and molecular analysis of *IFNGR2* revealed a homozygous dinucleotide deletion at nucleotides 278 and 279, resulting in a premature stop codon in the protein extracellular domain (Dorman and Holland, 1998). Clinical and immunological features were similar to those already described for complete IFN-γR1 deficiency.

Partial IFN-γR2 deficiency

Partial IFN-γR2 deficiency has been reported in a young adult with a history of curable infections due to *M. bovis* BCG and *M. abscessus*. The patient was homozygous for a nucleotide substitution in *IFNGR2* (C→T substitution at position 114) causing an amino acid substitution (Arg→Cys) in the extracellular region of the receptor. Cell surface IFN-γR2 was detected by flow cytometry and cellular responses to IFN-γ were impaired but not abolished. Transfection with the wild-type *IFNGR2* gene restored full responsiveness to IFN-γ (Doffinger *et al.*, 2000).

1.3.2iic Interleukin-12 p40 subunit (*IL12P40*)

IL-12 is composed of two chains (p35 and p40, encoded by separate genes on different chromosomes) and mostly secreted by dendritic cells and macrophages. It is a potent inducer of IFN-γ. Mutation in *IL12P40* has been identified in a child with disseminated BCGosis and salmonella infection whose parents were first cousins. A homozygous frameshift deletion of 4.4kb including two coding exons was identified which abrogated IL-12 production (Altare *et al.,* 1998d). This patient responded to treatment with recombinant IL-12.

1.3.2iid Interleukin-12 receptor β1 subunit (*IL12RB1*)

The IL-12 receptor consists of two chains (IL-12Rβ1 and IL-12Rβ2) they are synthesised at certain stages of NK cell and T cell differentiation. Mutations in the *IL12RB1* gene (de Jong *et al.,* 1998; Altare *et al.,* 1998a), result in a similar phenotype to the IL-12 deficiency described above. Interestingly, the patients with defects in the IL-12 pathway had less severe disease when compared with those with complete IFNγR deficiency. It appears that impaired secretion of IFN-γ is the mechanism that accounts for susceptibility to mycobacterial infection in patients with IL-12 deficiency.

Genetic mutations leading to complete deficiency of IFN-γR1, IFNγR2, IL-12 p40 or IL-12Rβ1 are rare events in nature leading to disseminated infection with poorly pathogenic mycobacteria and early death. However, the milder phenotype observed in partial deficiency of IFN-γR1 and IFN-γR2 raises the possibility that more common variants of these genes may be present in the general population (Fig. 1.5). Such polymorphism may account for part of the observed variation in susceptibility to more pathogenic mycobacteria such as *M. tuberculosis*.

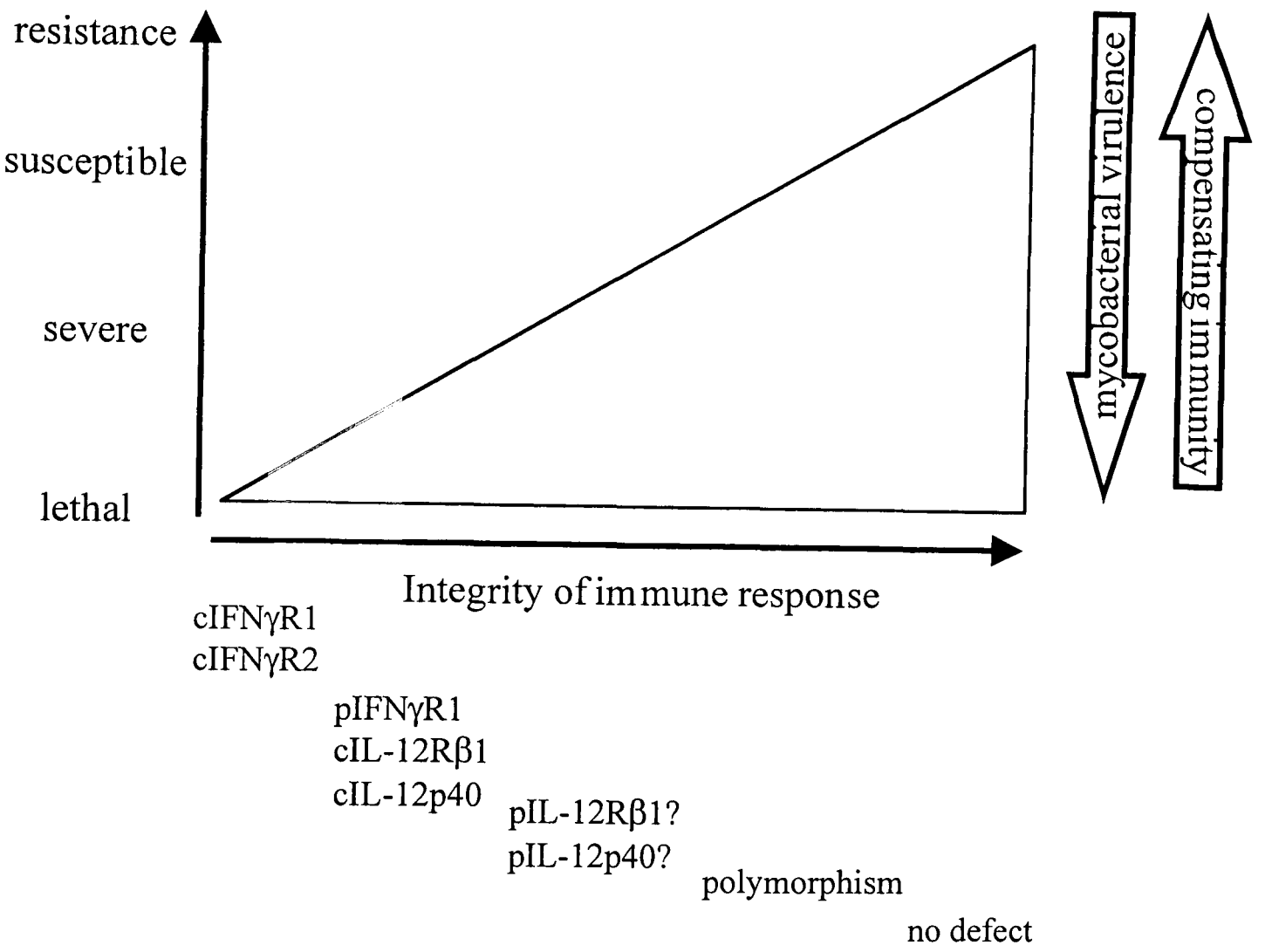


FIG. 1.5. Spectrum of genetic susceptibility to mycobacteria

Figure 1.5 illustrates the severity of mycobacterium infection in relation to host genetic defects, highlighting the importance of genetic regulation on the integrity of the ensuing immune response. At the extreme end of the spectrum are a few individuals with lethal disease such as was seen in the Maltese children, who suffered from atypical mycobacterium infection with an underlying single gene defect resulting in complete deficiency of the IFN-γ receptor. Individuals with partial deficiency of IFN- γ R, or complete deficiency of IL-12 p40 or IL-12R1β, have a milder phenotype. Although not yet reported, it is possible that partial deficiency of these latter proteins could result in a still milder phenotype, or polymorphism at the population level leads to a individuals who are more susceptible to more virulent pathogens. At the other extreme are resistant individuals with no genetic defect. (Figure 1.5 is derived from Ottenhoff et al., 1998)

1.3.2iii Identification of TB susceptibility genes in human populations

Infectious diseases such as TB are, from the genetic point of view, complex or polygenic traits. Inheritance does not follow the simple Mendelian recessive, dominant or sex linked pattern attributable to a single gene locus and the phenotype results from the interaction between a number of host genes, the environment and other factors. In the case of infection, disease also requires the presence of a pathogen, which has a genome too. It is not usually known exactly how many genes influence the phenotype, nor how they interact. Mutations in any one of several genes may result in an identical phenotype (genetic or locus heterogeneity), for example, when these genes are required for a common biochemical pathway such as macrophage activation. Although much progress has been made in mapping single genes disorders, the majority of diseases world-wide (e.g. heart disease, asthma, infectious diseases) are polygenic and identification of disease susceptibility genes remains a challenge (Lander and Schork, 1994). There is evidence from a Brazilian study that TB is under oligogenic control (Shaw *et al.,* 1997a).

There are two main approaches to identify human tuberculosis susceptibility genes. Firstly, one can look at genetic differences between individuals who have the disease compared to those who do not. This usually takes the form of an association study in which variation in a candidate gene is studied in cases and unrelated unaffected controls. Secondly, one can look for genetic similarities in family members who are affected (linkage studies). Family based linkage analysis can be used to screen the entire genome for chromosomal regions linked to disease, and therefore has the potential to identify novel genes. However, linkage analysis has limited power to detect genes with a modest effect. Subsequently large numbers of families are required in order to obtain a significant result (Risch and Merikangas, 1996). Furthermore, it is possible that an

individual has inherited the susceptibility gene(s), but is classified as unaffected due to lack of exposure to the pathogen. This can be overcome by studying affected family members (usually siblings) only. Candidate genes can also be studied by linkage, but a comprehensive search for regions associated with tuberculosis with a case control study design is currently not feasible for technical reasons. Association studies have the power to detect genes with a relatively small effect, but there are many potential candidates and the major susceptibility gene(s) may not yet have been discovered. Whereas linkage analysis requires families, association studies involve unaffected, unrelated individuals for comparisons. It is quicker to recruit study subjects for association (case control) studies than family members and siblings of affected individuals, providing a fast robust method of investigating the role of a particular gene in a disease. The statistical analysis is simple involving only chi square or 2 x 2 contingency table. In the long run, a combination of approaches is therefore likely to give the most success. Furthermore, family based association studies combine linkage and association for search for disease susceptibility gene.

Genome-wide searching for chromosomal regions linked to disease using affected sibling pairs, is currently popular as an approach to identifying disease susceptibility genes. The method is statistically robust as a genetic model is not required. Genome screens have been completed in several complex diseases. These include type 1 and type 2 diabetes (Davies *et al.,* 1994, Hanis *et al.,* 1996), inflammatory bowel disease (Hugot *et al.,* 1996, Satsagi *et al.,* 1996), multiple sclerosis (Sawcer at al., 1996), coeliac disease (Zhong *et al.,* 1996), asthma (Daniels *et al.,* 1996), and psoriasis (Trembath *et al.,* 1997). A genome wide search for TB susceptibility genes was carried out in Gambian affected sibling pairs and 5 regions were identified (Bellamy, 2000). However, although many regions have been identified as putative susceptibility loci for the various diseases, findings have not

been replicated in other studies, and genes have yet to be cloned. A number of association studies have now been reported for tuberculosis. Given its functional importance in immune responses to various antigens and infectious diseases, much effort has focused on the HLA region. Although associations have been demonstrated with both class I and II alleles, the majority have not been reproducible. At the population level the most consistent association is between tuberculosis and the HLA class II antigen *HLA-DR2* (e.g. Brahmajothi *et al.,* 1991).

Given the large amount of data in mice documenting the role of *Nramp1* in susceptibility to intracellular pathogens, the human homologue *NRAMP1* was a good non-HLA candidate gene for human susceptibility. Four *NRAMP1* polymorphisms were typed in a Gambian population and all found to be associated with tuberculosis (Bellamy *et al.,* 1998b). Variation in the vitamin D receptor was associated with resistance to tuberculosis (Bellamy *et al.,* 1998c) and a marginally significant association between tuberculosis and polymorphisms in the IL-1 gene cluster in the same Gambian population (Bellamy *et al.,* 1998d). So far, negative associations have been reported for the mannose binding lectin, fucosyltransferase-2 and IL-10 genes (Bellamy *et al.,* 1998d, 1998e).

1.3.2iv Quantitative phenotype analysis

Given the difficulties of mapping complex trait genes outlined above, many investigators have turned to quantitative trait analysis in order to simplify the problem. Quantitative traits exhibit continuous variation and include such measures as blood pressure, weight, serum cholesterol levels and immune responses. Many complex traits may be regarded as a constellation of quantitative traits, each having its own set of regulatory genes. By simplifying the phenotype, the likelihood of identifying these genes is theoretically enhanced. Quantitative trait loci (QTL) mapping has successfully identified susceptibility

genes for several traits in rodent models, including TB (Kreutz *et al.*, 1995, Lavebratt *et al.*, 1999).

This approach was adopted to identify human asthma susceptibility genes (Daniels *et al.*, 1996). A genome wide search for loci linked to eosinophilia, IgE levels, bronchial hyperresponsiveness and skin prick reactivity (all quantitative traits underlying asthma) identified 6 loci, one of which had been previously identified. As more is understood about the processes involved in the pathogenesis of TB, the quantitative traits underlying TB are increasingly amenable to genetic analysis.

1.4 Aims of this study

This study has investigated the role of specific candidate genes in immunity to mycobacteria and susceptibility to tuberculosis in Gambians (Table 5.1). One aim of the study was to determine whether the development of clinical TB was as a result of inferior ability to activate macrophage function. This study was expanded to search for evidence for correlation between cytokine secretion (as a marker of macrophage activation) and genetic variation. The next phase was to determine whether these genes influence susceptibility to TB. This study is the first to employ this approach towards identification of TB susceptibility genes in humans.

Candidate TB genes were selected based on what is known or suspected about their role in innate and acquired anti-mycobacterial immunity. Population based studies have reported associations between some candidate genes and clinical tuberculosis. Two recent studies of TB in Cambodia and India reported associations of some HLA class II alleles with TB (Goldfeld *et al.*, 1998, Ravikumar *et al.*, 1999). Also a study in Indian patients reported the influence of certain mannose binding protein alleles on TB (Selvaraj *et al.*,

1999). Previous study in The Gambia reported a predisposition of individuals by four NRAMP1 polymorphisms to TB (Bellamy *et al.,* 1998b). In the same population, an allele of the vitamin D receptor gene was associated with TB (Bellamy *et al.,* 1999). Polymorphisms in the cytokine genes IL-1β and its receptor antagonist IL-1RA were found to be associated with tuberculosis in patients of Gujarati origin living in England (Wilkinson *et al.,* 1999). The predisposing alleles reported in these studies have only moderate effects, and their functional relevance needs to be established before their role can be validated. Genes suspected to be important TB susceptibility genes include: - genes encoding macrophage deactivating cytokines interleukin 4, interleukin 9 and interleukin 10, macrophage-activating cytokines for example the TNF locus, the interferon gamma pathway, pathway for the generation of the free radical nitric oxide (NO) and the regions encoding the small inducible chemokine genes. Table 1.2 overleaf gives an overview of the candidate genes selected for this study according to its role in innate immunity to mycobacterial infection.

TABLE 1.2. TB candidate gene products

PATHWAY	CANDIDATE GENE PRODUCT
Macrophage entry by mycobacterium	Complement receptors1 and 3 Mannose receptor Fibronectin receptor
Macrophage activation	NRAMP1 Toll-like receptors CD14 molecule Vitamin D receptor
Antigen presentation	MHC T cell receptor TAP
Response to macrophage activation	Cytokines and their receptors IFNγ, TNF-, IL-1β IL-4, IL-8, IL-9, IL-10, IL-12, IL-13, CC Chemokines and their receptors :I-309, MCP-1, MIP-1α, MIP-1β, RANTES, MCP-3, MCP-2 Signal transduction proteins protein kinases such as JAK 1, JAK 2, and Stat 1α Nuclear transcription factors NF-kβ, IRF-1 NF-IL6, GAF.

This list contains an overview of genes that are involved in the macrophage activation process. This study concentrated on the innate immune responses to TB in humans and other genes important in acquired immune responses to TB were not selected.

CHAPTER 2

STUDY SUBJECTS,

MATERIALS AND METHODS

2.1 Recruitment of study subjects

The study subjects recruited for the studies described in chapters 3-6 can be divided into two groups - those with a history of TB (either current or past) and healthy individuals who had no history of TB. This project had the approval of the Gambian Government/Medical Research Council Joint Ethical Committee. Verbal consent was obtained from all patients before entry into the study. For logistical and sociological reasons (e.g. women rarely donate blood and present less frequently to medical services with TB), all subjects studies were male. All individuals were unrelated.

2.1.1. Subjects with a history of tuberculosis.

TB cases were recruited from two centres. Firstly, The Gambia National Tuberculosis and Leprosy Control programme runs a TB clinic at Serrekunda Health Centre near the capital, Banjul. Secondly, the Medical Research Council (Fajara) runs a chest clinic where newly diagnosed cases were recruited in to the study. All cases presented with typical clinical features of pulmonary TB and acid fast bacilli were identified in their sputa using standard Ziehl-Neelson staining. Ninety-five percent were culture positive by BACTEC, and most patients had X-ray changes suggestive of TB. HIV testing was carried out on all TB patients using an ELISA method established in the MRC Laboratories, Fajara and HIV positive individuals were excluded from the study. The prevalence of HIV infection in The Gambia is relatively low (below 2% in pregnant women, O'Donovan *et al.,* 2000) although the prevalence among TB patients is higher, as expected, at approximately 10% (Lienhardt *et al.,* 1999).

The study subjects with a history of TB could be further divided into those with a past history of TB (referred to as ex-cases) who had successfully completed the recommended

treatment regimen and been off treatment for at least two months, and those with TB at the time of enrolment. The former group (n=45) were used to address the hypothesis that innate defects of macrophage/monocyte function predispose to TB – since active *M. tuberculosis* infection itself and the drugs used to treat TB influence the immune response, subjects were recruited after successful completion of TB therapy. These subjects were traced using the Serrekunda TB clinic records, and interviewed by a trained field assistant to determine whether symptoms suggestive of TB were present. Those without such symptoms were recruited into the study. Symptomatic individuals were referred to the MRC clinic for further evaluation and not enrolled. The second group of cases were recruited for the case control genetic study from both Serrekunda TB clinic and the MRC TB clinic (n=276).

2.1.2 Healthy individuals

A major part of the work described in this thesis focuses on the genetic regulation of monocyte responses in healthy individuals who did not have active tuberculosis (chapters 3 and 5). Three hundred and twelve blood bank donors form the Royal Victoria Hospital Banjul were identified who did not have symptoms or signs suggestive of active tuberculosis, who were HIV negative, and who agreed to participate in the study. These subjects were used as controls for the TB cases in association genetic studies described in chapter 6.

A second group of healthy individuals were recruited as controls for the study investigating innate immune responses in ex-TB cases (n=45). These subjects were matched for age, ethnic group and drawn from the same locality as the ex-cases. An interview was conducted to exclude symptoms suggestive of active TB and symptomatic

individuals (including symptoms suggestive of other disease) were referred to the MRC clinic for further evaluation, and excluded from the study.

Demographic features of the study subjects are shown in tables 2.1 and 2.2, and figures 2.1 and 2.2. The mean age of TB cases was 35 years and the mean age for controls was 33 years.

2.1.3 Sample collection

Ten ml of venous blood was collected using an aseptic technique into 3.8% citrate, for macrophage function studies and for DNA extraction, as described in sections 2.3.1.i and 2.3.2.i below. For individuals in whom monocyte function assays were performed, 3 ml was placed in sterile eppendorf tubes. DNA was extracted from the remaining whole blood either immediately or after storage at –20° C. Two hundred and seventy six active TB cases were included for the case control association study in chapter six. Ten mls of venous blood was aseptically drawn into 3.8% sodium citrate and transported to the laboratories in Fajara. Macrophage function assays was not done on these samples. DNA was extracted for use in genotyping as described in section 2.3.2i.

Table 2.1 **Ethnic structure for case control association study (chapter six)**

Ethnic group	Controls	TB cases
Mandinka	104(0.33)	82(0.3)
Wollof	66(0.21)	35(0.13)
Jola	63(0.2)	60(0.22)
Fula	38(0.012)	49(0.18)
Serere	18(0.06)	20(0.072)
Manjagoe	3(0.0096)	15(0.054)
Serahuleh	5(0.016)	5(0.018)
Aku	3(0.0096)	3(0.01)
Bambara	5(0.016)	5(0.018)
Other	6(0.019)	2(0.007)
Total	**312**	**276**

Figures in parentheses represent frequency of the corresponding ethnic group

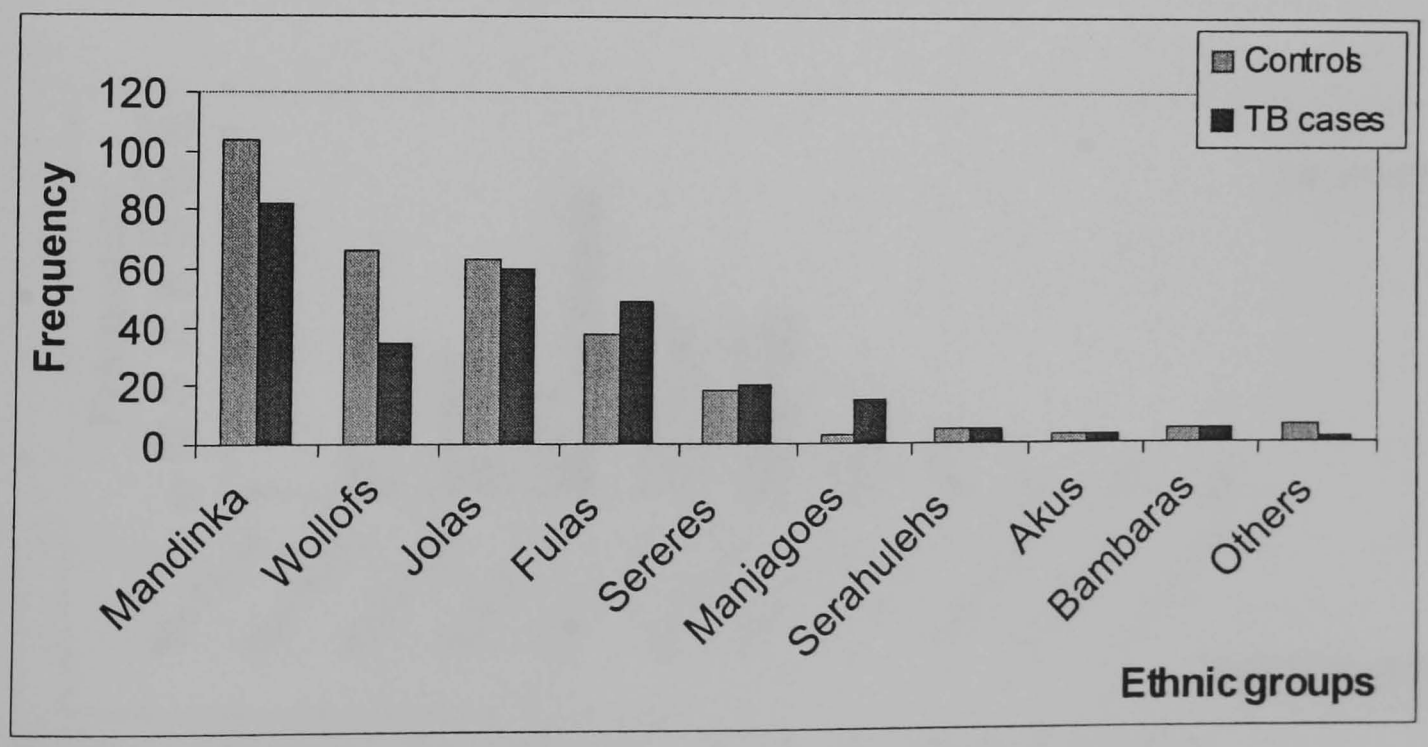
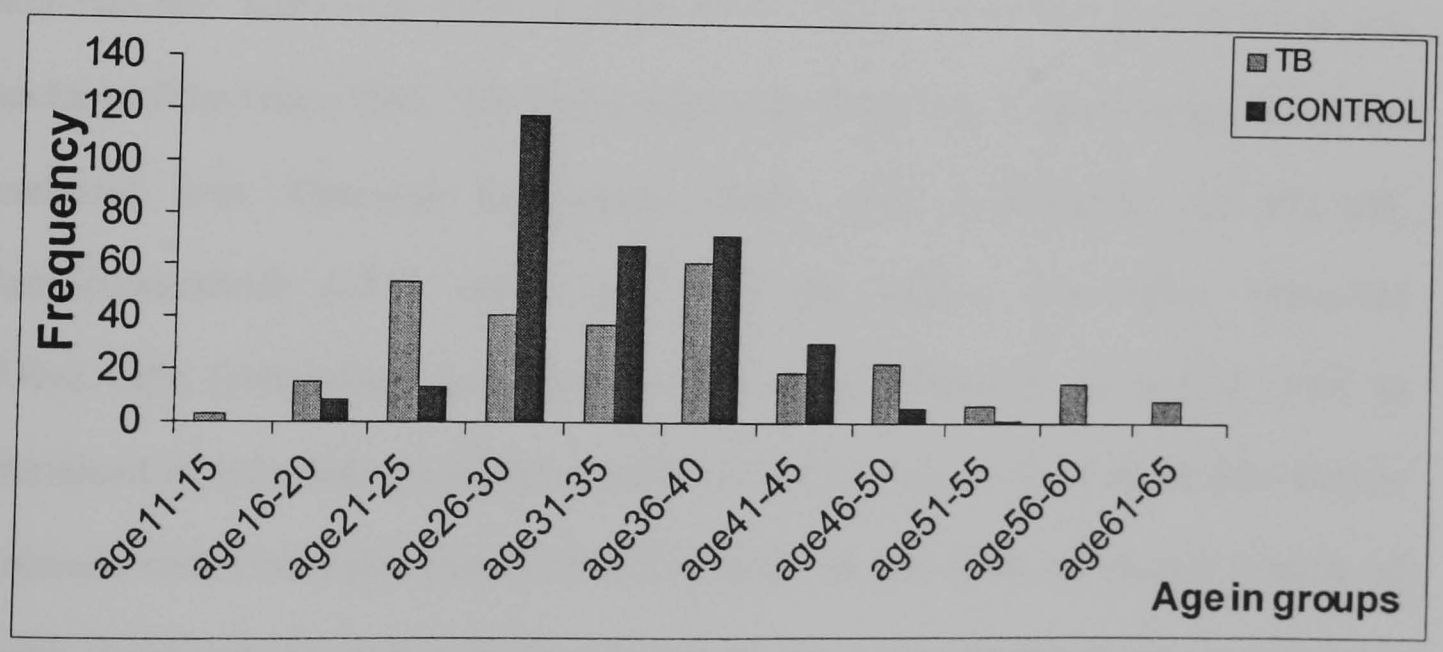


FIG.2.1 Ethnic groups represented in case control association study

Table 2.2 Age distribution of TB cases and controls

Age group (yrs)	Controls	TB cases
11-15	0 (0)	3(0.01)
16-20	9 (0.028)	15 (0.053)
21-25	13 (0.04)	54 (0.19)
26-30	118 (0.37)	41 (0.14)
31-35	68 (0.213)	37 (0.13)
36-40	72 (0.23)	61 (0.22)
41-45	31 (0.97)	19 (0.07)
46-50	6 (0.019)	23 (0.08)
51-55	1 (0.003)	7 (0.025)
56-60	0 (0)	15 (0.05)
61-65	0 (0)	9 (0.32)
Total	**312**	**276**



Figures in parentheses represent frequency of the corresponding age group

FIG. 2.2 Age distribution of TB cases and controls

2.2 Materials

2.2.1 Chemical reagents

Most of the chemicals and reagents used were purchased from Sigma Chemical Company (St Louis, MO). Trisodium citrate and Bis N, N' methylene bis acrylamide were obtained from British Drug Houses Laboratory Supplies (BDH, U.K.). Ultrapure deoxynucleotide triphosphates (dNTPs) and ficoll Hypaque were purchased from Amersham Pharmacia Biotech AB, (Uppsala, Sweden). Phenol (tris- equilibrated) was obtained from Gibco BRL Life Technologies (Scotland, U.K.). Commercial primers were purchased from either Gibco BRL Life Technologies (Scotland, U.K.) or Perkin Elmer Biosystems (Warrington, U.K.). Primers for the TNF ARMS PCR were from Cruachem Company (Glasgow, U.K.) and were gifts from Professor Kwiatkowski.

2.2.2 Immunology materials

Tissue culture medium RPMI 1640 was obtained from Whittaker (Walkersville, MD), endotoxin-free water was obtained from Braun (Belgium). Foetal bovine serum was purchased from Gibco BRL Life Technologies (Scotland, U.K.). Recombinant IFN-γ was purchased from Genzyme Diagnostics (Kent, UK). *Escherichia coli* 0111:B4, Lipopolysaccahride (LPS) was a gift from the Malaria Unit MRC. ManLAM 97.Rv2.4.25a (endotoxin concentration of 12 ng of endotoxin per mg of LAM as determined by quantitative Limulus amoebocyte assay) was provided by Dr John Belisle (Colorado state University, Fort Collins, Co) under the provision of National Institute of Health. Tetramethybenzedine, primary and secondary monoclonal antibody reagents for TNF, IL-1β, and IL-10 were obtained from BioSource International (Fleurus, Belgium). Streptavidin and horseradish peroxidase were purchased from Boehringer Mannheim

(Germany). Corning Laboratory Sciences Company (Corning, NY) supplied plastic ware unless otherwise stated.

2.2.3 Automated sequencing reagents

SequaGel concentrate and diluent for denaturing polyacrylamide gel electrophoresis used for sequencing was supplied by National Diagnostics (Hull, UK.) and Amresco Gene–PAGE Plus for microsatellite analysis was supplied by Amresco company (Solon, OH). Genescan 500-TAMRA, and 500-ROX (6-carboxy-rhodamine) size standard and fluorescently labelled primers were purchased from Perkin Elmer Biosystems (Warrington, UK.) The Wizard ^{TM} PCR preps DNA purification system and the fmol^{R} DNA sequencing system were purchased from Promega Corporation (Madison, USA).

2.2.4 Radioisotopes

[α ^{33}P] ATP as supplied by Amersham Pharmacia Biotech AB (Amersham, UK).

2.2.5 Enzymes

Taq DNA polymerase and Taq Gold were purchased from Perkin Elmer Biosystems (Warrington, U.K.). Biol X-act Taq and Buffer were obtained from Bioline (London, U.K.). Proteinase K was purchased from Boehringer Mannheim Biochemicals (Germany) and Gibco BRL Life Technologies (Scotland, U.K). Restriction endonucleases were obtained from New England Biolabs (Beverley, MA).

2.2.6 Electrophoresis

Agarose and DNA ladders (1 kb 100 bp and 123 bp) were supplied by Gibco BRL Life Technologies (U.K). Bis N, N' methylene bis acrylamide, acrylamide and NNN'N' tetramethylene diamine (TEMED) were supplied by Bio-Rad Laboratories Incorporated (Hercules, CA). 3mm chromatographic paper was purchased from Whatman limited (Clifton, NJ).

2.2.7 Photography and auto-radiography

Agarose gels were photographed using Polaroid 667 film from Polaroid (Cambridge, MA). Kodak X Omat K TM LS (24 X30 cm) used for autoradiography was obtained from Eastman Kodak company (New Haven, CT).

2.3 Methods

2.3.1 Immunological methods

2.3.1.i Monocyte function assay

Monocyte function was assessed using a whole blood assay and was carried out as already described (De Groote *et al.,* 1992, Levin *et al.,* 1995) with modifications. Briefly, one ml of whole blood was diluted with an equal amount of tissue culture medium RPMI 1640 (Whittaker Walkersville, MD) to achieve a _ dilution. Two hundred μl aliquots of diluted blood were stimulated in triplicate with either LPS (*Escherichia coli* 0111:B4, 1ng/ml) or manLAM, 2500ng/ml with or without pre-incubation with IFN-γ (Genzyme, 200U/ml) for 2 hours or in tissue culture medium (negative control). Plasma cytokine production at 18 hours of incubation in 5%CO_2 at 37^0C was measured by enzyme linked immunosorbent assay (ELISA).

2.3.1.ii Plasma cytokine levels by enzyme linked immunosorbent assay

Cytokine concentrations in plasma were measured using a one step antibody sandwich ELISA. Ninety-six well microtitre plates (Nunc^{TM} MaxiSorp F96, Scotland U.K) were coated with 100 μl per well (2 μg/ml) of high-grade anti-cytokine capture monoclonal of the cytokine being tested. Plates were stored overnight at 4^0C for adsorption. The contents of the wells were discarded and 200 μl of 1X PBS/1% bovine serum albumin were added for two hours at room temperature after which the contents of the wells were discarded and washed four times with 1X PBS/0.1% Tween. Fifty μl of diluted plasma was added to

each well followed by the addition of a detector biotinylated anti cytokine monoclonal antibody of the cytokine being measured at a concentration of 0.58μg/ml. Plates were incubated for 2 hours at room temperature with shaking. Recombinant cytokine was used for the standard and 1in 2 dilutions were made starting with 1000 pg/ml. Each plate was washed four times with 1X PBS/10% Tween. Streptavidin was conjugated to peroxidase in phosphate buffer solution (PBS) according to the specification of the manufacturer (Boehringer Mannheim, Germany). One hundred μl of the conjugated streptavidin horseradish peroxidase was added, the plates were incubated for another 30 minutes with shaking at room temperature. Wells were washed four times with 1 X PBS/Tween and drained. One hundred μl of tetramethylbenzidine (TMB) in TMB substrate buffer was added and the plates incubated at room temperature in the dark for 20 minutes. 50 μl of 2.5M sulphuric acid was added to stop the reaction and plates were read on a plate reader (Dynex Technologies, West Sussex, UK) at a wavelength of 450 nm to determine the cytokine concentration in each well relative to the standard. A standard curve was constructed by plotting the OD on the ordinate against the standard concentrations on the abscissa.

2.3.2 PCR Methodologies

2.3.2.i DNA extraction

For most samples DNA was extracted using a standard phenol chloroform extraction method. Briefly, cells were lysed using lysis buffer (0.32M sucrose, 10Mm Tris, 5mM $MgCl_2$, 1% Triton X100), the nuclei pelleted by centrifugation (3000 rpm for 15 minutes) and the supernatant was discarded. The nuclei pellet was re-suspended in 7.5 ml extraction buffer (10mM Tris pH 8.0, 0.1M EDTA pH 8.0 0.5% SDS) and incubated at 37°C for one hour. Proteinase K was added at a concentration of 100 μg/ml and the solution was incubated at 50°C for 3-18 hrs with periodic mixing. An equal volume of

phenol (3 ml) saturated with 100mM Tris HCl_2 pH 8.0 was added. This was rotated for 10 minutes and then centrifuged for 5 minutes at 3000 rpm at 20°C. The upper aqueous phase was removed and re-extracted further with 3ml phenol. The aqueous phase was removed and extracted with 3 ml of chloroform/isoamyl alcohol (24:1). To the aqueous phase, 9 ml of ice cold absolute ethanol was added in the presence of either 1.5 ml of 7.5M ammonium acetate or 3M sodium acetate (pH 5.2). DNA was hooked out with a glass pipette or pelleted by centrifugation. The DNA was dissolved overnight in 0.5 ml sterile distilled water. At other times the Nucleon kit was used for extraction according to the manufacturer's recommendations (Scotlab Scotland, U.K.).

2.3.2ii Optimising PCR conditions for novel polymorphisms

PCR conditions were first optimised in 20 µl master mix consisting of 1 µl of 50 ng/µl of each oligonucleotide, 2 µl of 10X reaction buffer, 2 µl of 2mM dNTP's, 13.9 µl distilled water, 0.1µl of 5 unit/µl of Taq polymerase. To optimise PCR conditions, different reaction buffers were used and their compositions can be found in appendix 1. Whichever, buffer gave the expected product size was then used in subsequent experiments. The final concentration of dNTP was 200µM. Primers and enzymes were stored separately from PCR products. Reactions were set up in a class II hood. Nucleotides, primers and enzymes were stored at –20°C. Buffers were prepared in house or were supplied by the manufacturer to accompany DNA polymerase enzymes. Magnesium ion concentration was optimised for each set of primers. Titration of magnesium chloride was achieved usually using a concentration of 0-10 mM free magnesium. Target DNA if in excess can give rise to non-specific amplification. In practice 100 ng of DNA was used per PCR reaction. The mastermix was mixed by vortexing and then aliquoted in 19 µl volumes into sterile 0.5-ml eppendorfs. All frozen stock solutions were mixed well before use. One µl of extracted genomic DNA (100

ng/μl) was added to each eppendorf tube and layered with 3 drops of mineral oil. A negative control was included for all experiments in which 1 μl of autoclaved distilled water was added instead of DNA. The tube was covered and spun briefly. The cycling parameters for each pair of oligonucleotide primer were first determined empirically using the guidelines described by Thein and Wallace (1986). The size of the amplified product was confirmed by gel electrophoresis through 2% agarose directly and visualised by UV light after staining the DNA with ethidium bromide. Gels were photographed using Polaroid 667 film.

2.3.2.iii Polyacrylamide gel electrophoresis

Glass plates were thoroughly cleaned with detergent and dried with paper towels. The inside surfaces of the plates were washed with Tepol detergent and left to dry for 2-3 minutes. Spacers were placed between the glass plates and the plates were clamped with bulldog clips. Gel mix was prepared for manual sequencing as follows: Bis acrylamide 0.3g, acrylamide 5.7g, Urea 4.8g, 10X TBE buffer 10 ml, Alpha Q water 45 ml. It was ensured that all the reagents were fully dissolved before the addition of ammonium persulphate (APS, 450 μl of a 10% solution) and TEMED (45 μl). The gel was poured between the glass plates. An inverted 48 well sharks-tooth comb was placed between the plates at the top of the gel. Bulldog clips were used to clamp the top of gel. Gels were left for a minimum of two hours to set before mounting on the M2 sequencing electrophoresis apparatus (Life Technologies, Inc.) When the gel had set, the comb was removed and the shark-tooth end reinserted to create wells into which samples were loaded prior to electrophoresis. For semi-automated sequencing and microsatellite analysis, gels were prepared as described above but using 40 μl of Sequagel concentrate and diluent for denaturing polyacrylamide gel electrophoresis and 40 ml (19:1 Acrylamide/Bis-acrylamide) Amresco Gene PAGE Plus respectively to which 200 μl of 10% APS and 20

µl of TEMED were added. Gels were mounted in an ABI automated sequencer for electrophoresis (Perkin Elmer, ABI-Prism 377).

2.3.2.iv Purification of PCR products

PCR products were purified prior to sequencing in order to remove contaminants such as primer-dimers and amplified primers. One hundred µl of PCR product was purified with the Promega wizard kit following the instructions of the manufacturer. The purified DNA was stored at 4^0 C. To confirm that the DNA has been purified a small amount of purified product was run on a 2% agarose gel before sequencing reactions.

2.3.2.v Manual sequencing

The fmol™ Promega sequencing protocol was employed according to the manufacturer's instructions. This involves the use of a thermal cycler and an enzymatic method initially developed by Coulson and Sanger (Sanger *et al.,* 1977). The method takes advantage of the intrinsic properties of the DNA polymerase, isolated from *Thermus aquaticus* (Taq DNA polymerase) and extension/termination reactions using direct incorporation of radioactivity are employed. For each individual, four micro-centrifuge (0.5 ml) tubes were set up for each sequencing reactions, one for each nucleotide, labelled A, C, G and T. To the appropriate tube, 2 µl of dideoxy and deoxy ribonucleotide triphosphate dNTP, ddNTP (A = Adenine, C= Cytosine, G = Guanine and T = Thymine) stock concentration was added. The tubes were capped and placed on ice or at 4^0C. For each set of sequencing reactions, the following reagents were mixed in a microcentrifuge tube: 9.5 µl of template DNA (33 ng/ µl), 1 µl of sequencing primer (50 ng/µl), 1 µl of [α- 33p] dATP (1000 Ci/mmol, 10 uCi / µl), 5 µl of 5 X fmol R sequencing buffer (250mM Tris-HCl_2 pH 9.0 at 25 ° C, 10 mM $MgCl_2$). A control reaction consisting of 5µl of PGEMR-3Zf (+) control DNA (500 fmol) and, 2.5 µl of PUC/M13 forward primer (3 pmol), 1 µl of [α-^{33}P] dATP (1000Ci/mmol, 10µCi/µl), 5 µl of 5X fmol R sequencing buffer were used instead of

template DNA and sequencing primer. To the primer/template mix, 1.0 µl of sequencing grade Taq DNA polymerase (5U/µl) was added and the contents mixed by pipetting. Finally, to the inside wall of each tube containing d/ddNTP, 4 µl of enzyme primer/template mix was added. One drop of mineral oil was added to each tube, which was then spun briefly in a microfuge. The MJ thermal cycler was pre heated to 95°C and the cycling program was started. A thermal cycle profile recommended by the manufacturer for cycle sequencing was used thus: - 95°C for 2 minutes, 30 cycles of 95°C for 30 seconds, 42°C for 30 seconds and 70°C for 1 minute. After the thermal cycling program was completed, 3 µl of fmol sequencing stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% Xylene Cyanol FF) was added to the inside wall of the each tube. The product was spun briefly in a microcentrifuge to terminate the reaction. The sequencing reaction was either processed immediately or stored at -20^0C for a maximum of four days before being analysed. The reaction was heated at 70°C for 2 minutes immediately before loading on a 6% polyarcylamide sequencing gel. Three µl of each reaction mixture was loaded on the gel. After electrophoresis, the gel was transferred onto Whatman paper, dried, and subjected to autoradiography.

2.3.2.vi Semi automated Sequencing

Semi automated sequencing was carried out in Professor Dominic Kwiatkowski's laboratory in the Institute of Molecular Medicine (IMM), University of Oxford. Genomic DNA extracted using a standard phenol chloroform method as described in section 2.3.2.i was used as template for amplification. The procedure employed involved an initial primary PCR amplification of the region to be sequenced (in this case IFN-γ and IFN-γ R1 gene promoter regions) with subsequent secondary amplification using M13 tailed primers. Thirty-six samples were sequenced using the ABI PRISM ™ BigDye™ Primer Cycle Sequencing Ready Reaction Kit, following the instructions of the manufacturer.

Briefly, secondary PCR products were diluted 1:6 to achieve DNA concentration of 100 ng/μl. Ninety-six well PCR plates were labelled accordingly (A, C, G, T) and a 0.5X reaction master mix was prepared as described in the manufacturers manual, consisting of 2μl of each nucleotide concentrations and 2μl of milliQ water respectively. One μl of diluted secondary PCR product was then added. Mineral oil was layered over the reaction to avoid evaporation. The plate was spun at 1700 rpm for 2 minutes after which thermal cycle sequencing was performed using the GeneAmp 9600 protocol or 2400 thus: - An initial 20 times cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 70°C for 1 minute and additional 15 cycles of 96°C for 10 seconds and 70 °C for 1 minute. Reactions ACGT were pooled for each individual into one labelled tube and spun at 13000 rpm for 1 minute to separate the reaction mixture from the mineral oil. Again, the sequencing reaction was either processed immediately or stored at –20°C for a maximum of four days before being analysed. Fifty-three μl of absolute ethanol was added to a fresh microfuge tube and the sequencing reaction was transferred into the tubes without taking any mineral oil. This was left to stand for ten minutes at room temperature before spinning at 13000 rpm for 15 minutes in the microfuge. After centrifugation ethanol was aspirated from the opposite side of the hinge so as not to disturb the pelleted DNA. The pellet was left to dry and was resuspended in 4 μl of loading dye. DNA was denatured at 95°C for 3 minutes and loaded into individual wells of a polyacrylamide gel using the ABI PRISM 377 apparatus. The DNA sequence was analysed using the sequence navigator programme.

2.3.2.vii Microsatellite analysis

Microsatellite analysis was carried out in Professor Adrian Hill's laboratory, (Oxford, a microsatellite identified in the interferon-γ receptor-1 gene) and in Professor Jennefer Blackwell's laboratory (Cambridge, U.K.). Primers used to amplify a panel of ten

macrophage candidate genes were designed to create PCR products of different sizes without overlap between the first and last alleles of different markers (Appendix 2, table 2.1) PCR conditions for eight primers had been optimised in the laboratory. Two (IL-10 and IFN-γ) were added and optimised during the course of this study. One primer of each pair was labelled with a 2'7'-dimethoxy-4', 5'-dichloro-carboxy fluorescein compound at the 5' end. Three fluorescein dyes were used FAM™ for blue, NED™ for yellow and HEX™ for green. PCR conditions were optimised for each primer pair independently. This was done in 5 μl volumes to include primers used at concentrations as described in chapter six, 2.5μl of 20ng/μl DNA, ABI buffer supplied by the manufacturer, 2.5mM each of dNTPs, 1.5 mM $MgCl_2$ and 0.15U of Amplitaq Gold polymerase. PCR reactions were carried out as stated in chapter six using an MJ research thermal cycler. Products were pooled in a standard way such that the product labelled with FAM were at half the concentration of those labelled with other dyes. The products were added to 2.5μl of deionised dimethyl formamide, 0.5μl of bromophenol blue running buffer (Applied Biosystems) and 0.5μl of a fluorescein labelled ROX standard (Applied Biosystem). This was electrophoresed through a 6 % polyacrylamide gel at 900 Volts on an ABI 377 DNA Sequencer (Applied Biosystems). The alleles were detected by an argon ion laser beam that scans across the gel at a fix distance from the sample loading wells and analysed using GENESCAN ™ and Genotyper 2.2 software (Applied Biosystems). The microsatellite PCR product sizes were determined by reference to the internal lane ROX size standards (Carrano *et al.,* 1989). Alleles were globally binned by use of a simple computer program and numbered in order of size and results were presented in a spreadsheet format (Appendix 2, table 2.1).

2.3.2.viii Single nucleotide polymorphism (SNP) analysis

Single nucleotide polymorphisms (SNPs) in candidate genes of interest were analysed using two methods.

2.3.2.viii.a Restriction fragment length polymorhism (RFLP) analysis

DNA regions containing SNPs of interest were amplified by PCR and the amplicons incubated with restriction endonucleases. The presence of the SNP wither created or abrogated a restriction enzyme site. PCR was carried out in 12.5 µl volume with primer concentrations as stated in chapter six. The PCR conditions were as follows: -6.25µl of 50ng/µl of DNA, 160mM $(NH_4)_2SO_4$, 670mM Tris–HCl_2 pH 8.8 at 25 $^{\circ}$C 0.1% Tween, 2.5mM of each dNTP, 1.5 mM $MgCl_2$ and 0.15U of Bioline Taq polymerase. After PCR amplification of a segment of DNA containing a SNP, the amplified product was subjected to enzyme digestion. If there is a restriction enzyme site, the endonuclease cuts the PCR product. Because, there are two chromosome homologues, the homozygote wild and mutant genotypes and the heterozygote genotype were easily differentiated by electrophoresis of the digested DNA through a 2% agarose gel, which was stained with ethidium bromide and visualised under UV light. Ten candidate genes were genotyping by PCR-RFLP analysis (Appendix 2, table 2.2).

2.3.2.viii.b Amplification refractory mutations system (ARMS) PCR

Amplification refractory mutation system technique (ARMS-PCR) can detect single base changes, or insertions or deletions of a few nucleotides resulting in variation in DNA sequence. It requires three primers designed for each mutation. Allele-specific primers complementary to the wild-type or the mutant sequence, which differ only in their 3' nucleotide (according to the SNP present in the gene of interest) are used with a consensus primer for each SNP typed. Two PCR reactions are set up for each individual, which contain the consensus primer and either of the allele-specific primers. The presence

of a mismatch at the 3' end of a primer prevents amplification during PCR. The genotype of an individual is determined according to which primer pairs lead to amplification. Control primers designed to amplify a different part of the genome are incorporated in to the PCR mix to ensure that non-amplification at a given locus is due to the absence of a particular allele, rather than PCR failure. Standard PCR was carried out as follows: - each reaction mix of 12.5µl contained 50ng/µl DNA, 670mM Tris- HCl_2, 160mM $(NH_4)_2$ SO_4, 2mM $MgCl_2$, 100uM of each dNTP, 0.1 % Tween-20, 0.5U of Taq polymerase. The touch down procedure was employed for all ARMS-PCR carried out. This involves an initial denaturation at 96°C for 1 minute followed by five cycles of denaturation at 96°C for 35 seconds. Annealing was at 70°C for 45 seconds and elongation for 35 seconds at 72°C. Twenty-one cycles of denaturation at 96°C for 25 seconds, annealing at 65°C for 50 seconds then elongation for 40 seconds at 72°C, followed. Finally, additional six cycles of denaturation at 96°C for 35 seconds, annealing at 55°C for 1minute and elongation for 1minute 40 seconds at 72°C followed. The presence or absence of the desired band was resolved by running PCR product through a 1.5-% agarose gel stained with ethidium bromide and visualised under UV light. Four polymorphisms in TNF gene were genotyped using this method (see section 5.3 and appendix 2, table 2.3).

2.3.2.ix Variable number of tandem repeats (VNTR) analysis

Standard PCR was performed as described for RFLP analysis above. PCR was carried out in 12.5µl volume with primer concentrations as stated in chapter six respectively, 6.25µl of 50ng/µl of DNA, 160mM $(NH_4)_2SO_4$, 670mM Tris $-HCl_2$ pH 8.8, 0.1% Tween–20, 2.5mM of each dNTP, 1.5 mM $MgCl_2$ and 0.15U of Bioline Taq polymerase. The size variations of VNTR alleles were large enough to be differentiated on a 1.5% agarose gel. Polymorphisms in the IL-4 and IL-1 receptor antagonist genes were genotyped using this method (see section 5.3 and appendix 2, table 2.4).

2.4 Statistical analysis

An EXCEL package was used to calculate the concentration of cytokines from OD of ELISA data in plasma samples described in chapter 3. Analysis of data was performed using Sigma plot version 1. For cytokines measurements, background production was subtracted from antigen-stimulated production. Data were logarithm transformed to achieve a normal distribution before t test was used for comparisons between groups. Spearman's rank and pairwise correlation coefficient was used to calculate the correlation co-efficient for LPS and LAM data in chapter 3. Spearman's method assigns a rank to each observation in each group separately in contrast to the Kruskall Wallis rank sum method were ranks are pooled and then calculates the sums of the squares of the differences in paired ranks (d_x^2) according to the formula below.

$Rs = 1\text{-}6\ (d_1^2 + d_2^2 + d_3^2 +\quad d_x^2)/(n(n^2\text{-}1).$

The Kruskall Wallis one way analysis of variance for non parametric continuous data was used to test the hypothesis that the mean cytokine measurement differ among genotypes. The data are ranked and the sampling distribution is arranged so that it follows the chi square distribution as follows:

$\text{Chi-square} = 12 / N\ (N+1)\ (R^2_o/ n_o + R^2_1/n_1 + R^2_2/N_2 +\quad + R^2_x/N_x) - 3(N+1)$

Where N = total number of observations, x = number of groups, R_o = the rank sum of group o. This statistics is compared against the χ^2 with N-1 degrees of freedom, and if it exceeds the value corresponding to the alpha level the null hypothesis is rejected. This test was used to determine the difference in cytokine induced responses between genotypes in chapter 5. A p- value below 0.05 was considered significant. Please refer to chapter 6 for detail description of the method used for correction multiple testing.

All statistical analysis was done using Stata v5 (Stata Corporation, College Station, Tx, USA).

CHAPTER 3

STUDIES OF MONOCYTE FUNCTION

3.1 Introduction

Macrophage activation is a key event in the immune response to intracellular pathogens including *M. tuberculosis.* Several stimulants such as bacterial cell wall components can activate macrophages. Macrophage activation results in the up regulation of MHC class II molecules, production of reactive oxygen and nitrogen intermediates and the release of cytokines, for example IL-1β, IL-10 and TNF necessary for effective containment of infection (see section 1.2.1i). Activation of the macrophage is enhanced by the Th1 type cytokine interferon-gamma (IFN-γ), produced by T cells and NK cells. Evidence from studies of 'knockout' mice (Cooper *et al.,* 1993) and children deficient in IFN-γR1 (Newport *et al.,* 1996) have clearly highlighted the importance of this pathway in resolving intracellular infections. In-vitro assays of monocyte/macrophage function (for example the production of macrophage cytokines in response to certain stimuli) can be used to assess responsiveness to IFN-γ. For example, a whole blood assay was used to determine a lack of IFN-γ responsiveness in a group of Maltese children with IFN-γ R1 deficiency (Levin *et al.,* 1995).

This method assesses monocyte function in whole blood by measuring cytokine release (e.g. TNF) as a result of mononuclear cell stimulation with LPS or LAM and can also assess the responsiveness to IFN-γ (Wilson *et al.,* 1991). The whole blood model has been applied to study T cell mediated immunity in leprosy (Weir *et al.,* 1994), human leishmaniasis (Frankenburg and Klaus, 1991), IFN-γ in TB (Takashima *et al.,* 1990) and to the immune response to BCG in neonates using mycobacterial antigens (Marchant *et al.,* 1999a). This method has also been applied to study cytokine regulation, for example IL-10, in healthy people (Eskdale *et al.,* 1998). The whole blood assay is a functional assay that gives minimal intra-individual, day-to-day variation and is therefore a useful phenotype used to define the role of genetic variation. A comparison between whole

blood assays and those performed on isolated peripheral blood mononuclear cells showed that whole blood assays exhibited less day-to-day variation and were more reproducible (De Groote *et al.,* 1992). The whole blood cell culture is an elegant ex-vivo technique that keeps the blood microenvironment intact and avoids the extraction procedure required for PBMC isolation that is associated with modification of cell ratios and activation. It requires a smaller blood volume, is relatively rapid and simple and therefore applicable to field studies.

Pro-inflammatory cytokines such as TNF, IL-1β and anti-inflammatory cyokines such as IL-1RA and IL-10 are well-characterised cytokines that are easily detected by enzyme linked immunosorbent assay (ELISA). Microbial cell-wall components such as LPS from gram-negative bacteria (Wilson *et al.,* 1991) and manLAM from mycobacteria can be used to stimulate whole blood to produce these pro and anti-inflammatory cytokines.

The aim of the study was to find out whether individuals who develop TB in The Gambia have innate defects in macrophage function, determined by responsiveness to IFN-γ and subsequent release of cytokines after stimulation with bacterial cell wall components. This study was based on the hypothesis that pathways of monocyte activation are abnormal in patients who develop disease as a result of infection with *M. tuberculosis*. Cellular studies were carried out using a whole blood model in patients with TB in The Gambia.

Studies were designed to investigate monocyte function in healthy individuals. Initially an in house whole blood assay was optimised; optimised conditions were then used to test the hypothesis that individuals who are susceptible to TB have defects in their innate immunity, demonstrated by defects in monocyte activation. Thirty subjects were included in the study. Inclusion criteria for selection into the study have been described in section 2.1.1 and 2.1.2. Secondly, the assay was used to evaluate variation in monocyte responses

in a healthy population and to generate immune data for subsequent genetic studies aimed at identifying genetic factors influencing monocyte responses.

3.2 Methods

3.2.1 Study population

Ten healthy Gambians (healthy research centre staff) were recruited for the optimisation of the whole blood assay. For the recoverd-TB versus control study, a sample size of 40 individuals were presumed sufficient in each group to have 80% power to detect a 25% difference in the mean production between the groups at a significance level of 95%. The TB cases all had microscopically confirmed TB and at the time of study had completed treatment at least two months previously and were asymptomatic. This allowed the study of innate immune responses that were not affected by *M. tuberculosis* disease or anti-tuberculosis chemotherapy both of which influence immune response (Takashima *et al.*, 1990). In order, to investigate the relationship between the immune response and host genotype, a larger sample size was required. Three hundred and sixty (360) healthy blood donors were recruited in to this study. If an allele at a frequency of 0.2 increases cytokine production by 25%, a sample size of 350 is required to detect this at 80% power and 95% significance.

3.2.2 Optimisation of whole blood assay conditions.

Optimal conditions necessary for the release of cytokines in The Gambian population had to be established. These conditions were ascertained by determining the effect of varying the following parameters: Blood dilution factor, IFN-γ concentration, LPS concentration, manLAM concentration and the length of culture.

Three ml of whole blood from each healthy volunteer was drawn by venepuncture into 3.8% citrate. Whole blood has been reported to contain inhibitory substances that may

affect cytokine responses. Whole blood was therefore diluted to achieve 1 in 2 and 1 in 8 dilutions with 10% fetal bovine serum enriched endotoxin-free RPMI 1640 tissue culture medium (Bio Whittaker) and cultured with different concentrations of LPS, manLAM and recombinant IFN-γ. Final concentrations of 0 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml, and 100 ng/ml of LPS, and, 1 ng/ml, 10 ng/ml 100ng/ml and 2,500 ng/ml of man LAM were used. A titration of IFN-γ concentration was also performed for each dose of LPS as follows: 0 U/ml, 50 U/ml, 100 U/ml, 200 U/ml, 400 U/ml, 800 U/ml of IFN-γ. Based upon the LPS results, the optimal concentration (200 U/ml) of IFN γ was subsequently used for studies with LAM. Co- and pre- rIFN-γ priming of whole blood was carried out to determine the effect of IFN-γ on TNF production. Whole blood was either pre-incubated with 200U/ml rIFN-γ for 2 hours at $37^{O}C$ in $5\%CO_2$ prior to the addition of LPS and LAM, or it was added simultaneously. A time course assay was also carried out by harvesting supernatants at 3hrs, 5hrs, 8hrs and 18hrs after the addition of either LAM or LPS. Cytokine levels were measured using an antibody sandwich ELISA as described in section 2.3.1i.

3.2.3 Finalised whole blood assay protocol

Based on the results of the experiments described above the following method was used to study monocyte function in both studies described. Typically, 9 ml of venous blood was collected into 3.8% citrate. One ml was centrifuged immediately and plasma was separated. This was used to determine baseline cytokine levels. Two ml of blood was diluted 1: 2 with foetal bovine serum supplemented tissue culture medium (RPMI 1640): 2 ml of diluted blood was incubated with rIFN-γ (200U/ml) and another 2 ml without rIFN-γ for 2 hours in $5\%CO_2$ at $37^{0}C$. The blood was then stimulated with either LPS or manLAM in 200μl triplicates in a 96 well plate, with a final concentration of 1 ng/ml LPS and 2500 ng/ml LAM. In addition, two negative control wells were done for each sample,

without LPS and LAM or IFN-γ. Blood was then incubated for 18 hours at 37^{O}C in 5%CO_2. Supernatants were collected into corresponding positions in a new 96 well plate. The production of IL1β and TNF (pro-inflammatory) and IL-10 (anti-inflammatory) cytokines, was measured using commercially available ELISA kits as already described in section 2.3.1ii. ManLAM was unavailable at the start of these experiments and was therefore not used for the study of TB and matched controls.

3.3. Results

3.3.1. Optimisation of assay

The following conditions were observed as optimal for cytokine release from ten individuals used for the optimisation assay. Data presented is the mean cytokine concentration of these 10 individuals. Assays were carried out in triplicate per individual, when cytokine concentration was similar in all wells, the mean of the triplicate was calculated per individual and when there was a great difference between wells, then the mean concentration of two wells with similar results was used. Usually cytokine concentration was considered to be similar if they were only 5% apart. Mean TNF levels did not differ considerably whether 1 ng/ml or 10 ng/ml of LPS was used (Table/Figure 3.2) nor for whether 200, 400, or 800U of IFN-γ. The following concentrations of stimulants were therefore selected for of TNF release: 2500 ng/ml LAM (Table/Figure 3.1) and 1 ng/ml of LPS and 200 U/ml of IFN-γ (Table/Figure 3.2).

Table 3.1 Mean TNF levels induced by LAM in the presence of rIFN-γ

LAM conc.	LAM 1/2	LAM 1/10	LAM+IFNγ 1/2	LAM+IFNγ 1/10
1ng/ml	444	1276	214	1388
10ng/ml	526	1619	186	1121
100ng/ml	452	1536	334	1039
1000ng/ml	1042	2405	1661	5651
2500ng/ml	1310	3110	2049	9120

Table 3.1 shows the mean TNF levels (pg/ml) induced by different concentrations of LAM in the presence or absence of a fixed concentration (200U) of rIFN-γ. Whole blood was diluted with tissue culture medium RPMI 1640 supplemented with foetal bovine serum to obtain either a 1 in 2 or a 1 in 10 dilution and was primed with rIFN-γ. TNF levels are in picograms per millilitre. The 1 in 10 dilution of whole blood induced higher TNF levels whether priming with rIFN-γ was done or not. Please refer to appendix 3 table 3.1 for the raw data for the generation of mean TNF concentration induced by LAM in the presence of rIFNγ.

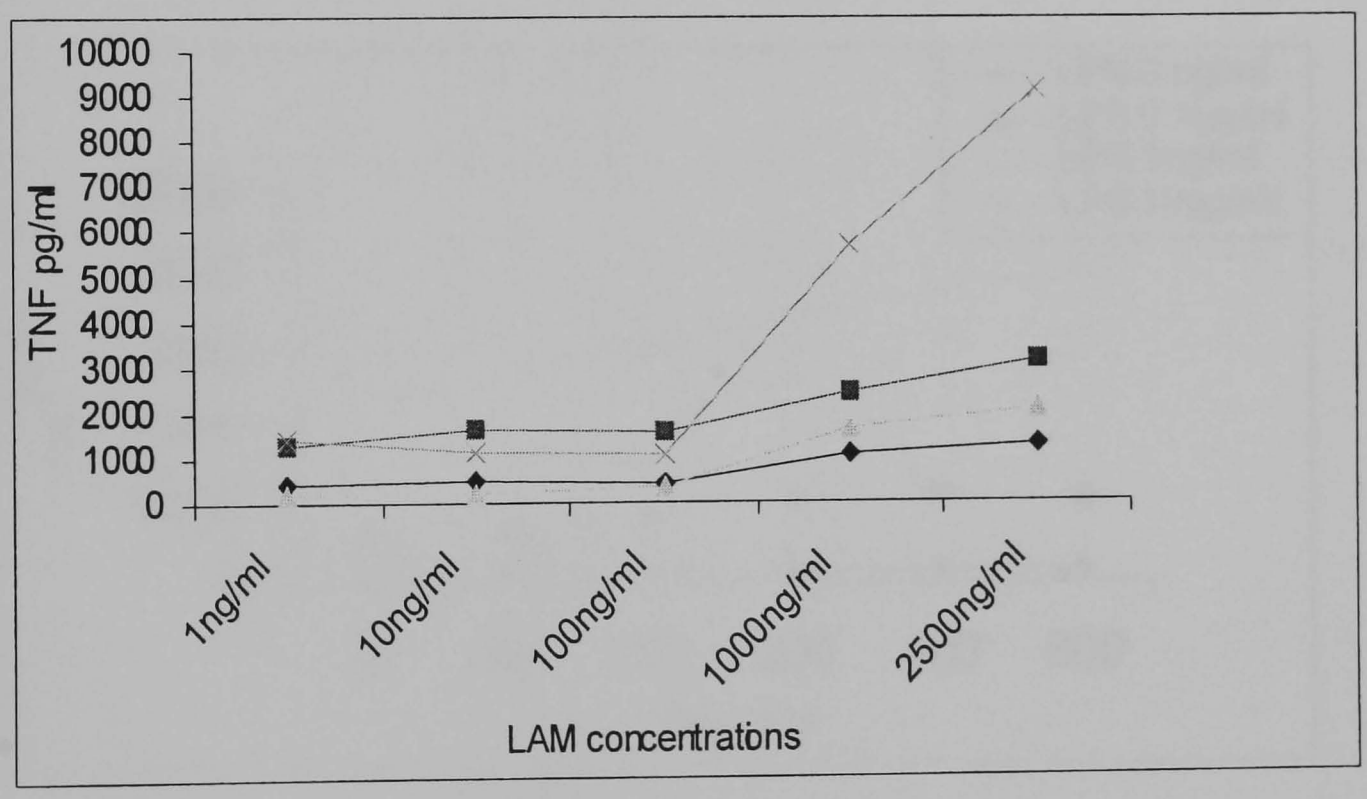


FIG.3.1 Mean TNF levels induced by LAM in the presence of rIFN-γ.

Figure 3.1 shows the mean TNF levels in response to either LAM alone or LAM and IFNγ. LAM concentrations were varied. IFN-γ was used at 200 IU/ml. Whole blood was diluted 1 in 2 and stimulated with either LAM alone (closed diamonds) or pre incubated with rIFN-γ for 2 hrs prior to the addition of LAM (closed triangles). Whole blood was diluted 1 in 10 and stimulated with either LAM alone (closed squares) or pre incubated with rIFN-γ prior to addition of LAM (crossed lines).

Table 3.2 Mean TNF concentration induced by LPS

IFNγ Concentration in IU/ml						
LPS Conc.	0	50	100	200	400	800
LPS 0 ng/ml	118	215	256	247	296	322
LPS 0.1ng/ml	1112	1398	1780	2052	2219	2113
LPS 1ng/ml	3738	4862	5657	5178	6298	6356
LPS 10ng/ml	4980	5161	5987	6761	7420	7908

Table 3.2 gives mean TNF levels did not increase appreciably after stimulation with 1 ng/ml and 200 IU/ml of rIFN-γ. The data for the generation of mean TNF concentration induced by LPS in the presence of rIFN-γ can be found in appendix 3, table 3.2.

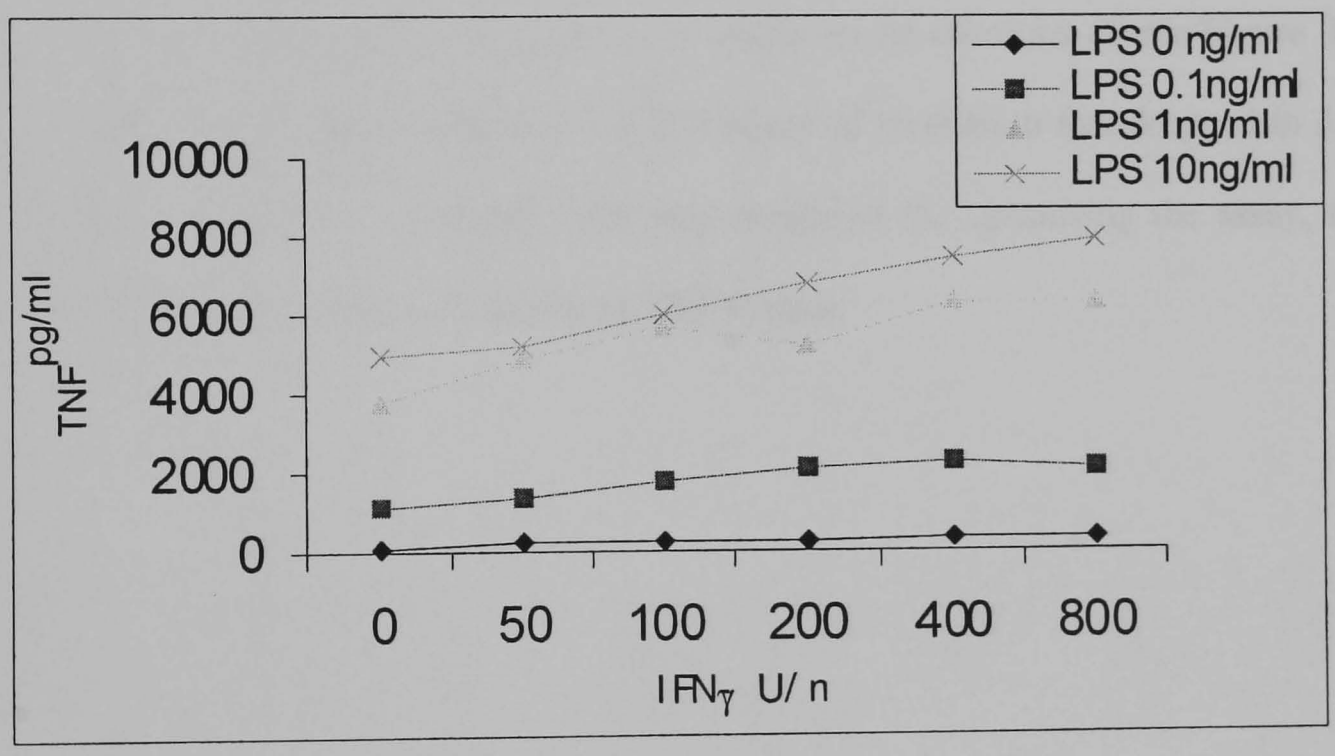


FIG. 3.2 Mean TNF levels induced by LPS in the presence of rIFN-γ

Figure 3.2 shows different concentrations of IFN-γ were used. Wholeblood was primed with IFN-γ alone without addition of stimulants (closed diamonds). Wholeblood was pre incubated with different concentrations of rIFN-γ prior to stimulated with 0.1ng / ml of LPS (closed squares), 1 ng / ml LPS (closed triangles) and 10 ng / ml LPS (crossed lines).

Cytokine production was optimal after 18 hours of incubation of whole blood with either LAM or LPS (Table/Figure 3.3 & 3.4). The average index of IFN-γ up-regulation (the ratio between the amount of TNF produced with IFN-γ priming to TNF produced without IFN-γ priming) was highest (4.5) after 18 hours of pre-incubation (Table/Figure 3.4) whereas after 18 hours of co-incubation TNF ratios declined to 1.58 (Table/Figure 3.3). The highest index of IFN-γ up-regulation after co IFN incubation was seen to be 2.28 at 8 hours of incubation (Table/Figure 3.3). Enhanced production of TNF was seen in most samples when whole blood was pre stimulated with rIFN-γ (Table/Figure. 3.3). The 1:2 dilutions gave better average index of up-regulation of 7.49 after 18 hours of incubation (Table/Figure.3.4) compared to the 1: 8 whole blood dilutions (Table/Figure 3.4) with average index of up-regulation of 3.8 at 8 hours of incubation that dropped to 3 after 18 hours of incubation. Although TNF was measured for optimising the assay, there are reports that IL-1β release is similar to TNF release.

Table 3.3 Ratio of TNF induced by co and pre IFN-γ incubation with LPS (1 ng/ml)

Length of incubation	Co- IFN-γ incubation	Pre-IFN-γ incubation
3 hours	1.33	1.7
5 hours	1.93	2.08
8 hours	2.28	2.74
18 hours	1.58	4.15

Table 3.3 gives the ratio of TNF induced by co IFN-γand pre IFN-γ incubation with LPS. At eight hours of incubation there was no significant difference between co- and pre IFN-γ incubations for IFN-γ up-regulation. After 18 hours of incubation IFN-γ up-regulation of TNF levels dropped for co-incubation of IFN-γ with LPS, whereas up-regulation of TNF levels continued to increase for pre IFN-γ incubation. The data for the generation of co and pre IFN- γ incubation with LPS can be found in appendix 3, Table 3.3.1 and 3.3.2.

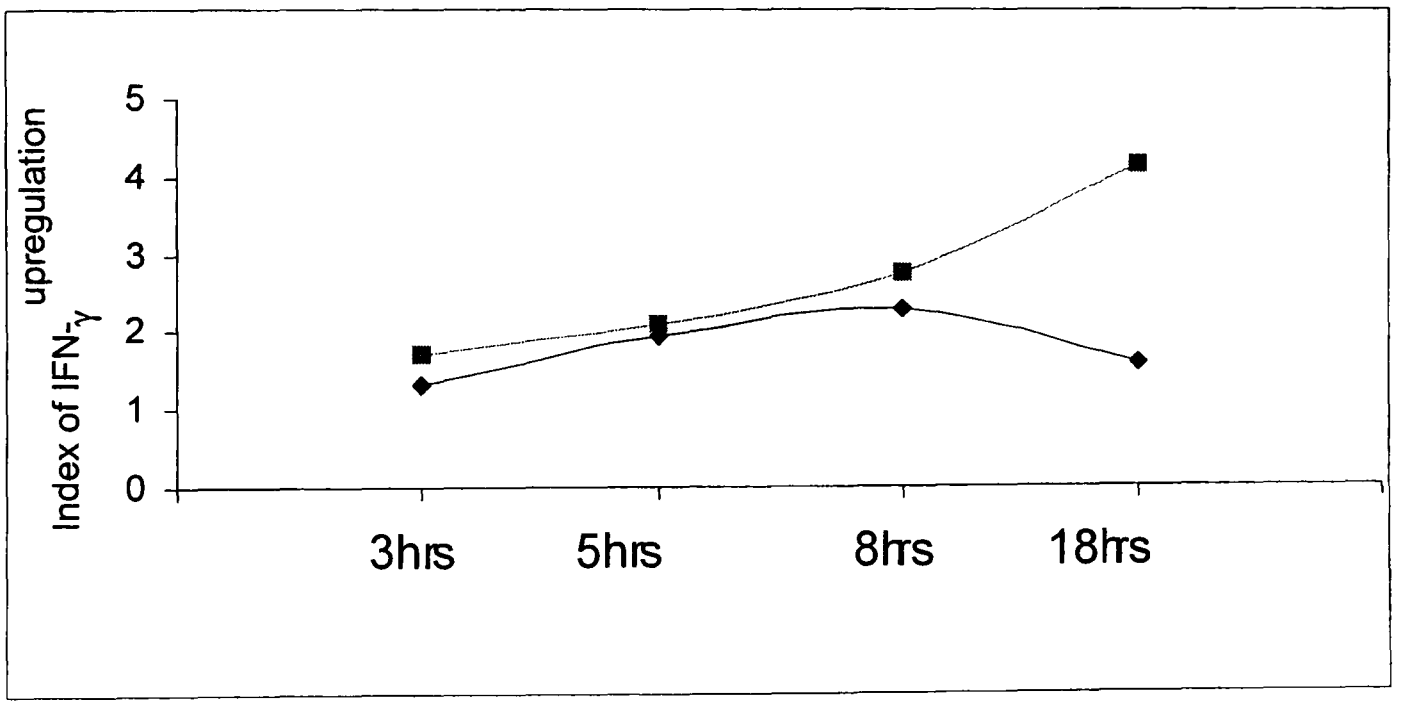


FIG.3.3 Ratios of TNF induced in response to co- and pre IFN-γ incubation with LPS.

Figure 3.3 shows the ratios of TNF induced in responses to co IFN-γ and pre IFN-γ incubations with LPS. Incubation of whole blood with rIFN-γ together with LPS, gave the average index of IFNγ up regulation of 2.5 after 8hrs of incubation and this dropped to 1.5 after 18hrs incubation (closed triangles). Whereas when whole blood was pre-incubated for 2hrs with rIFN-γ and later with LPS, the average index of up regulation was 4.5 after 18 hrs of incubation (closed squares).

Table 3.4 Ratio of optimal TNF release for 1 in 2 and 1 in 8 whole blood dilution

Length of incubation	Dilution factor	
	1 in 2 dilution	1 in 8 dilution
3 hours	4.64	3.12
5 hours	5.73	3.4
8 hours	7.08	4.55
18 hours	7.49	3.8

Table 3.4 gives a ratio for TNF release after dilution of whole blood. Whole blood was diluted to give either 1 in 2 or 1in 8 dilution. The 1:2 dilutions gave a better index of IFN-γ up-regulation for TNF release of 7.49 compared to 3.8 of 1 in 8 dilution after 18 hours of incubation. The data for the generation of ratio of optimal TNF release can be found in appendix, 3 table 3.4.1 and 3.4.2.

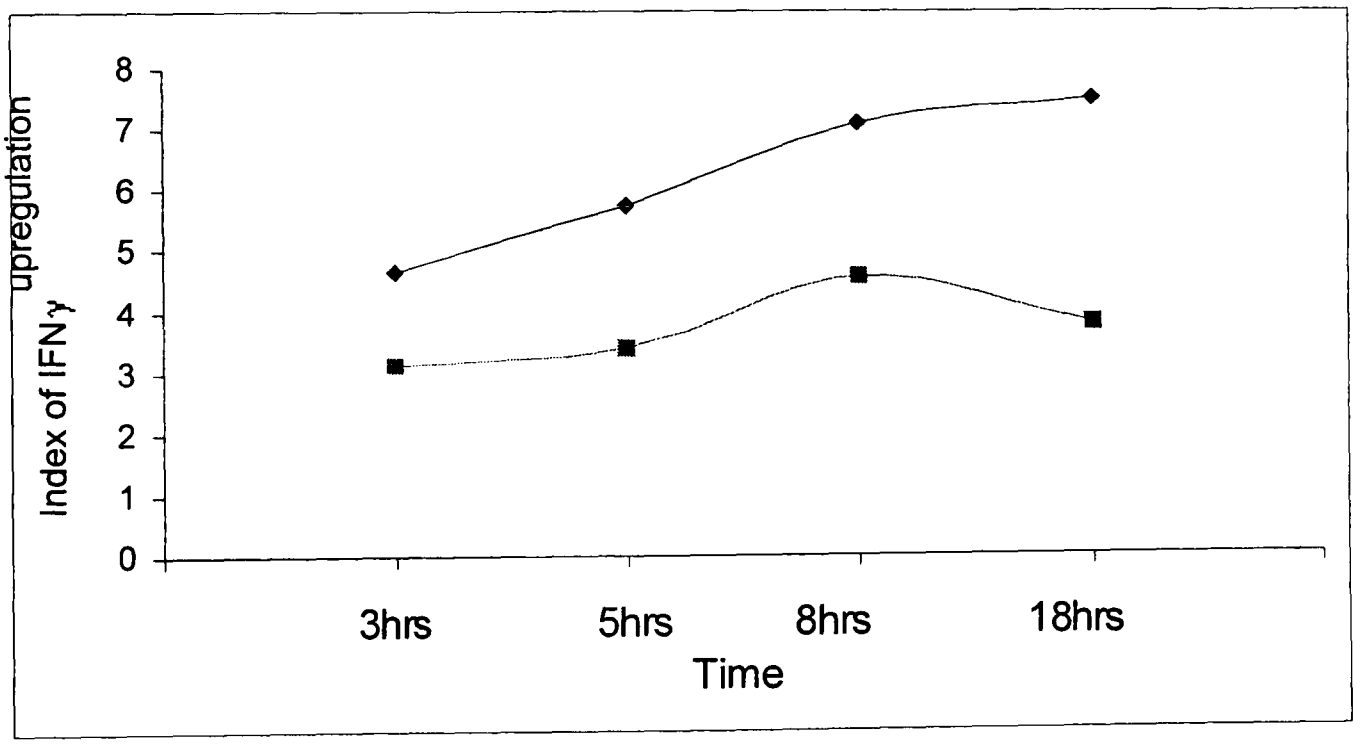


FIG.3.4 Effect of 1 in 2 and 1 in 8 whole blood dilution on TNF production

Figure 3.4 shows the effect of whole dilutions. Whole blood was diluted with foetal bovine serum supplemented RPMI 1640. The pre-rIFN-γ incubation for 2 hrs before addition of LPS was adopted. Average index of rIFN-γ up regulation for 1 in 2 dilution was 7.0 after 18 hrs of incubation (closed diamonds). Average index of rIFN-γ up regulation for 1 in 8 dilution was 4.0 after 8 hrs of incubation and dropped to 3.0 after 18hrs of incubation. The raw data for the generation of optimal TNF release can be found in appendix 3 table 3.4.1 and 3.4.2.

From the preliminary optimisation assay for cytokine release the following conditions were considered as optimal for TNF release: whole blood dilution of 1:2 with foetal bovine serum supplemented RPMI 1640, pre-incubation of whole blood with 200 U/ml of rIFN-γ, stimulation of whole blood with either 1 ng/ml of LPS or 2500 ng/ml of manLAM and eighteen hours incubation time after addition of stimulants. The 2 hours pre-incubation of whole blood with IFN-γ (200 U/ml) has been reported by other workers to enhance pro-inflammatory cytokine induction (Renz *et al.*, 1988, Rook *et al.*, 1987). Therefore these conditions were used during the course of this study. After establishing optimal conditions for cytokine release in this population, whole blood assays were performed in forty-five recovered TB cases and healthy controls as outlined in section 3.3.2. Further, cytokine measurements were carried out in an additional three hundred and twelve blood donors also outlined in section 3.3.3.

3.3 2. Whole blood studies in recovered TB cases

Cytokine levels were compared between recovered TB cases and healthy controls. TNF, IL-1β and IL-10 production was measured in response to LPS using the optimised whole blood assay described above.

Figures 3.5.1 and 3.5.2 overleaf show cytokine production by controls and ex-cases respectively, by individual. There are four bars per individual. Individuals have been represented as sample numbers. The first bar represents TNF production upon LPS stimulation; the second bar represents TNF induced upon priming of whole blood with IFN-γ prior to addition of LPS. The third bar represents IL-10 production and the fourth bar represents IL-1β production. There were no differences in mean cytokine production between ex-cases and controls. However, there was inter-individual variation in cytokine production. Some individuals were low responders for all cytokines typical and examples

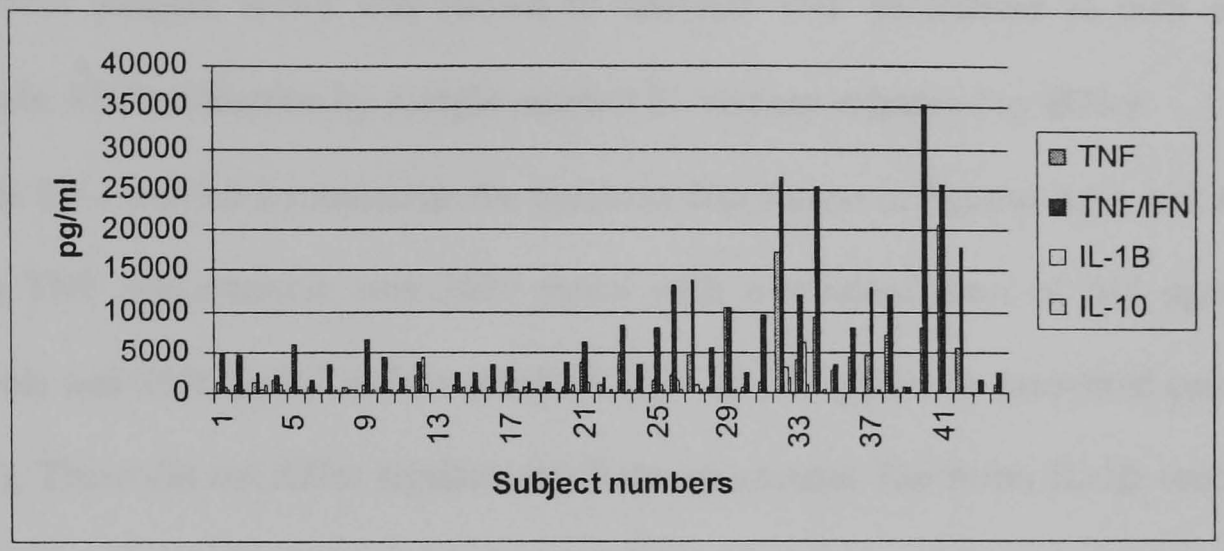


FIG. 3.5.1 Cytokine production in controls

Figure 3.5.1 shows heterogeneity of cytokine responses to LPS in different control individuals. For some of the assays there were technical problems and cytokine measurements was not done.

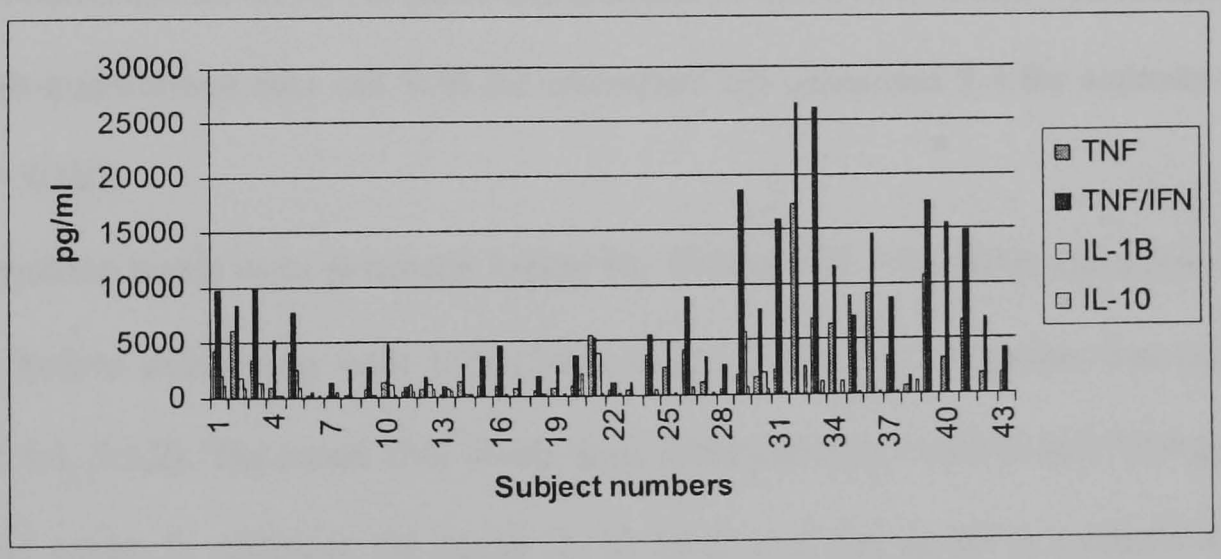


FIG. 3.5.2 Cytokine production in recovered TB cases

Figure 3.5.2 shows hetrogeniety of cytokine respones in recovered TB cases in response to LPS. Three cytokine responses are shown per sample. The first bars shows TNF induced by LPS alone the next bar shows TNF induced by LPS in the presence of IFN-γ followed by IL-1β responses and lastly by IL-10.

are sample number 8, 13, 18, and 19 for controls and sample number 6, 7, 11 and 13 for the ex-cases.

For most samples IFN-γ was shown to enhance TNF production in both cases and controls. TNF production by sample number 21 was not enhanced by IFN-γ.

Tables 3.5.1 and 3.5.2 summarise the cytokine data shown in Figures 3.5.1 and 3.5.2. The mean TNF concentration was 3029 pg/ml with a standard error of 647 pg/ml in the controls and 2896 pg/ml with a standard error of 543 pg/ml in recovered cases (Table 3.5.1). These did not differ significantly between groups. The mean IL-1β concentration was 834 pg/ml with a standard error of 263 pg/ml in controls and 994 pg/ml with a standard error of 282 pg/ml in cases. Again there was no statistically significant difference between groups. Mean IL-10 levels in recovered cases was observed to be higher (527 pg/ml with standard error of 90 pg/ml) than in controls (319 pg/ml with standard error of 56 pg/ml). Results for mean IL-10 between groups was significantly different between recovered TB cases and controls (Table 3.5.1, 3.5.2). The means of the logarithm-transformed data are 5.78 for recovered TB cases and 5.4 for controls (Table 3.5.2, $p= 0.02$).

Mean cytokine levels were generally higher for TNF and IL-1β release upon prior IFN-γ priming before stimulation with LPS (Table 3.5.1, 3.5.2). IL-10 release was repressed (Table 3.5.1, 3.5.2). The mean TNF levels were 7400 pg/ml in controls and 7155 pg/ml in recovered cases. In addition, the mean IL-1β level was 794 pg/ml in controls and 955 pg/ml in recovered cases. Mean pro-inflammatory cytokine levels were not significantly different between the two groups even upon IFN-γ priming prior to LPS stimulation (Table 3.5.1, 3.5.2).

Table 3.5.1. Cytokine production by controls and recovered TB cases in response to stimulation.

CYTOKINES	MEAN ± SEM in pg/ml (range)			
	CONTROLS		CASES	
	LPS	LPS & IFNγ	LPS	LPS & IFNγ
TNF	3029 ± 647 (100-20480) n=44	7400 ± 1434 (405-26563) n=44	2896 ± 543 (140-17270) n=43	7155 ± 1001 (420-26563) n=43
IL-1β	834 ± 263 (30-6032) n=28	794 ± 233 (57-4983) n=28	994 ± 282 (30-5610) n=25	955 ± 265 (62-6233) n=25
IL-10	319 ± 56 (32-1765) n=38	175 ± 28 (32-761) n=37	527 ± 90 (46-2494) n=38	282 ± 68 (30-1504) n=38

Table 3.5.1 gives the mean and standard error cytokine release in picograms per ml. The figures in parentheses are the minimum and maximum observed values. n is the number of individuals studied using each stimulant. There was no significant difference in TNF and IL-1β cytokine levels between recovered cases and controls. Appendix 4.1, 4.2 & 4.3 gives data for cytokine levels for each individual from which data were derived. The data was skewed (not normally distributed), they were log transformed for statistical analysis and presented in table 3.5.2

.

Table 3.5.2 Cytokine production by controls and recovered TB cases in response to LPS (Logarithm transformed)

CYTOKINES	MEAN±SEM pg/ml			
	CONTROLS		CASES	
	LPS	LPS & IFNγ	LPS	LPS & FNγ
TNF	7.23 ± 0.18**❋ n=44	8.36 ± 0.16❋ n=44	7.27 ± 0.19**+ n=43	8.34 ± 0.16+ n=43
IL-1β	5.8 ± 0.25** n=28	6 03 ± 0.19 n=28	5.95 ± 0.3** n=25	6.11 ± 0.25 n=25
IL-10	5.4 ± 0.79*✈ n=38	4.79 ± 0.72✈ n=37	5.78 ± 1.04*σ2 n=38	5.15 ± 0.93σ2 n=38

Table 3.5.2 shows the natural logarithm transformation of data in Table 3.5.1. Data was not normally distributed and was transformed to allow for t test analysis. IL-10 levels differed significantly between recovered cases* and controls*, p=0.02, IFN-γ repressed IL-10 levels in both cases (σ2) and controls (✈) p = 0.007. There was no significant difference in TNF and IL-1β levels between the two groups of recovered cases** and controls** p=0.4. However, the effect of IFN-γ up regulation on TNF in recovered cases and controls was statistically significant❋, + p=<0.00001.

Marked inter-individual variation was observed within both groups in the ability to up-regulate cytokine production in response to IFN-γ (Figures 3.6.1 & 3.6.2). By expressing the TNF production following IFN-γ priming as a ratio of the levels produced by LPS alone, we can calculate an index of IFN-γ up-regulation for this variability in responsiveness. Figures 3.6.1 and 3.6.2 shows the index of IFN-γ up-regulation in controls and recovered TB cases respectively. There are three bars per individuals. Individuals have been represented as sample numbers. The first bar represents the index of IFN-γ up-regulation for TNF α, the second bar represents the index of IFN-γ up-regulation for IL-1β and the third bar represents the index of IFN-γ up–regulation for IL-10. Marked heterogeneity of IFN-γ priming was observed in both groups (ranging from 0.9 to 11.8 fold increase, Figures 3.6.1 & 3.6.2).

Generally, TNF was up regulated by IFN-γ in both controls and recovered cases. Certain individuals were poor IFN-γ up-regulators of all three cytokines: this was observed more in the recovered cases than the controls. For example controls with sample number 20, 21 and 22 and recovered cases with sample numbers 1, 2, 3 and 4. The index of IFN-γ up-regulation was higher in controls for both TNF and IL-1β. For example seven control individuals had ratios > 6 whereas only two recovered cases had ratios > 6. However, the mean up-regulation of responses to IFN-γ priming did not differ significantly between the two groups (3.5 in the ex-cases and 4.0 in the control groups ($p=0.22$)). Generally, levels of IL-1β were lower than TNF. Based on the amount of TNF produced in response to IFN-γ priming following LPS stimulation, individuals could further Inter-individual variation in the amount of IL-1β produced was observed, and up-regulation of IL-1β production in response to IFN-γ occurred, but was less marked than for TNF (Table 3.6).

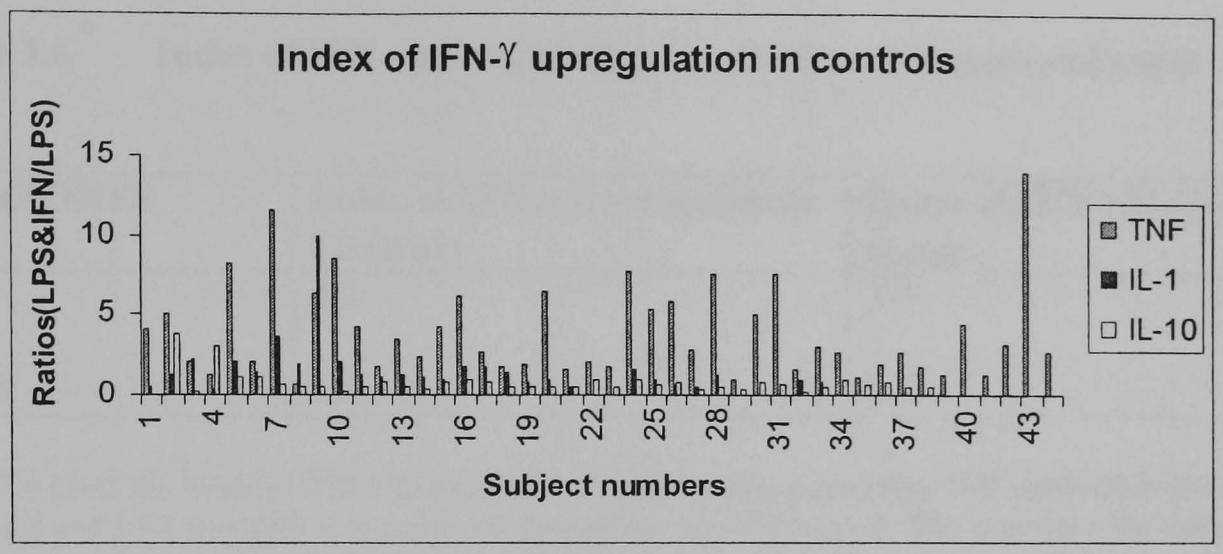


FIG. 3.6.1 Index of IFN-γ upregulation in controls

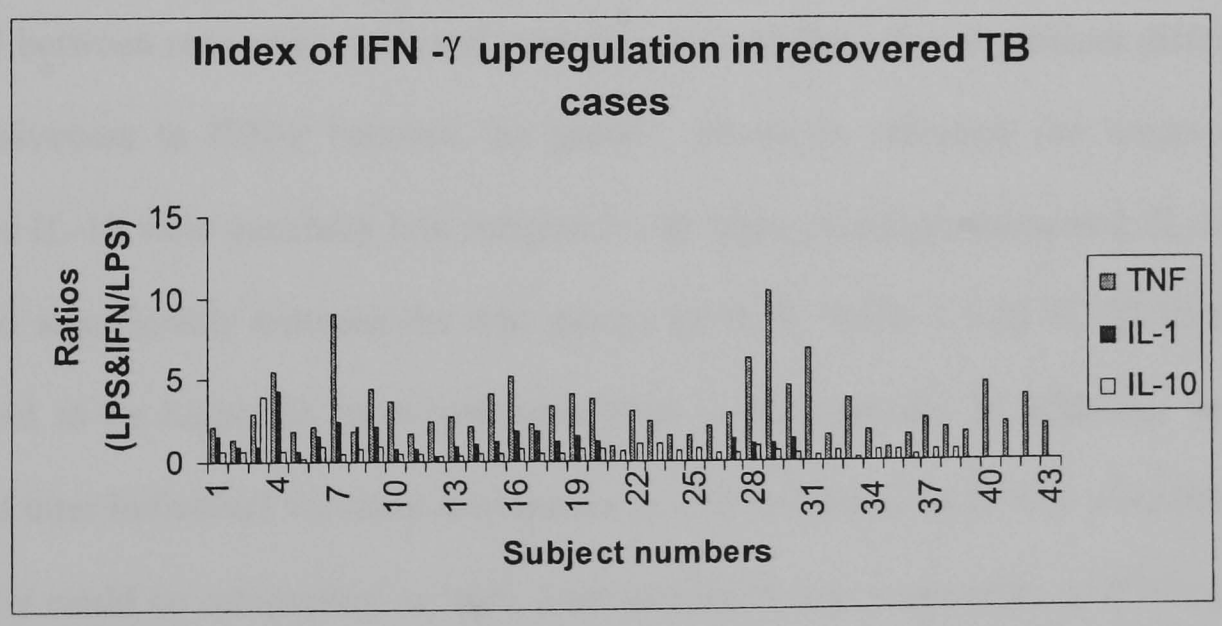


FIG. 3.6.2 Index of IFN-γ upregulation in recovered TB cases

Levels of IL-10 were generally lower than the pro-inflammatory cytokines measured. There was no IFN-γ up-regulation of IL-10.

Table 3.6 Index of IFN-γ up-regulation in controls and recovered cases

CYTOKINES	Index of IFN-γ Up-regulation Controls	Index of IFN-γ Up-regulation Cases
TNF	4.0	3.5
IL-1β	1.6	1.4

Table 3.6 gives the index of IFN γ up regulation is calculated by expressing TNF production following IFN-γ priming and LPS stimulation as a ratio of stimulation with LPS alone. This was done for each individual and each cytokine measured. IL-10 was not up regulated by IFN-γ; therefore the index of IFN-γ up regulation of IL-10 was not included in the table 3.6 above.

In conclusion, there was no significant difference in pro inflammatory cytokine responses to LPS between recovered cases and controls: nor was there any significant difference in responsiveness to IFN-γ between the groups. However, although the levels of LPS induced IL-10 were generally low compared with other cytokines measured, IL-10 levels differed significantly between the two groups ($p=0.02$, Table 3.5.2). IL-10 levels were observed to be higher in recovered cases than in the controls. In addition, there was marked inter individual variation in response to LPS and individuals who were responsive to IFN-γ could be categorised as high, intermediate or low responders to IFN-γ priming. IL-10 levels were rather repressed by priming with rIFN-γ (Tables 3.5.1 and 3.5.2).

3.3.3 Cytokine responses to LPS and manLAM in healthy individuals.

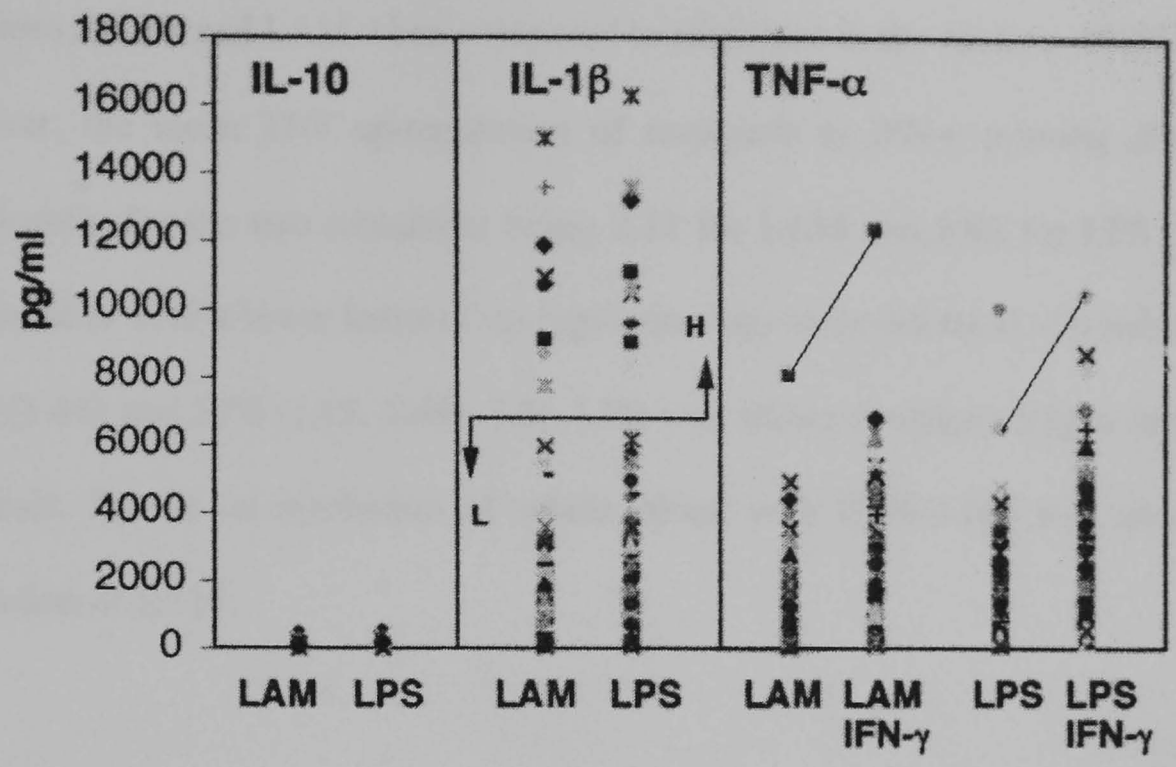
Further whole blood assays using a larger sample size was necessary to understand the mechanisms involved in the observed inter-individual variation in levels of cytokines. Three hundred and twelve healthy blood donors were studied in this study.

Again, there was considerable variation in the levels of cytokines produced by different individuals as expected given the findings in the study of recovered TB cases and controls described above. The mean TNF levels induced by LAM (1050 pg/ml, standard error of 92 pg/ml) did not differ significantly from that induced by LPS (911 pg/ml standard error of 67 pg/ml). In addition, the amount of TNF produced in response to LPS correlated with the amount produced in response to LAM (FIG.3.7.1). This was also true for IL-1β produced in response to LPS and LAM (Table 3.7). Mean IL-1β induced by LAM was 1749 pg/ml standard error of 245 pg/ml and that induced by LPS was 1405 pg/ml standard error of 142 pg/ml. Also, there was a positive correlation between TNF and IL-1β production in response to either LPS (FIG.3.7.2) or LAM (Table 3.9). This may suggest that the same genes or pathways regulate the responses to LPS and LAM, and the same genes may regulate the production of TNF and IL-1β. IL-10 levels induced by both LAM and LPS were generally low (66 pg/ml and 55 pg/ml respectively).

Mean cytokine levels were comparable between LPS and LAM stimulated cells on pre-incubating with IFN-γ (Table 3.7). Mean TNF levels were 1749 pg/ml for IFNγ primed LAM induced responses and 1758 pg/ml for IFN-γ primed LPS induced responses. In addition, the mean IL-1β level was 1846 pg/ml for IFN-γ primed, LAM-induced responses and 1666 pg/ml for IFN- γ primed LPS induced responses.

CYTOKINE PROFILE IN GAMBIAN CONTROL INDIVIDUALS

a)



b)

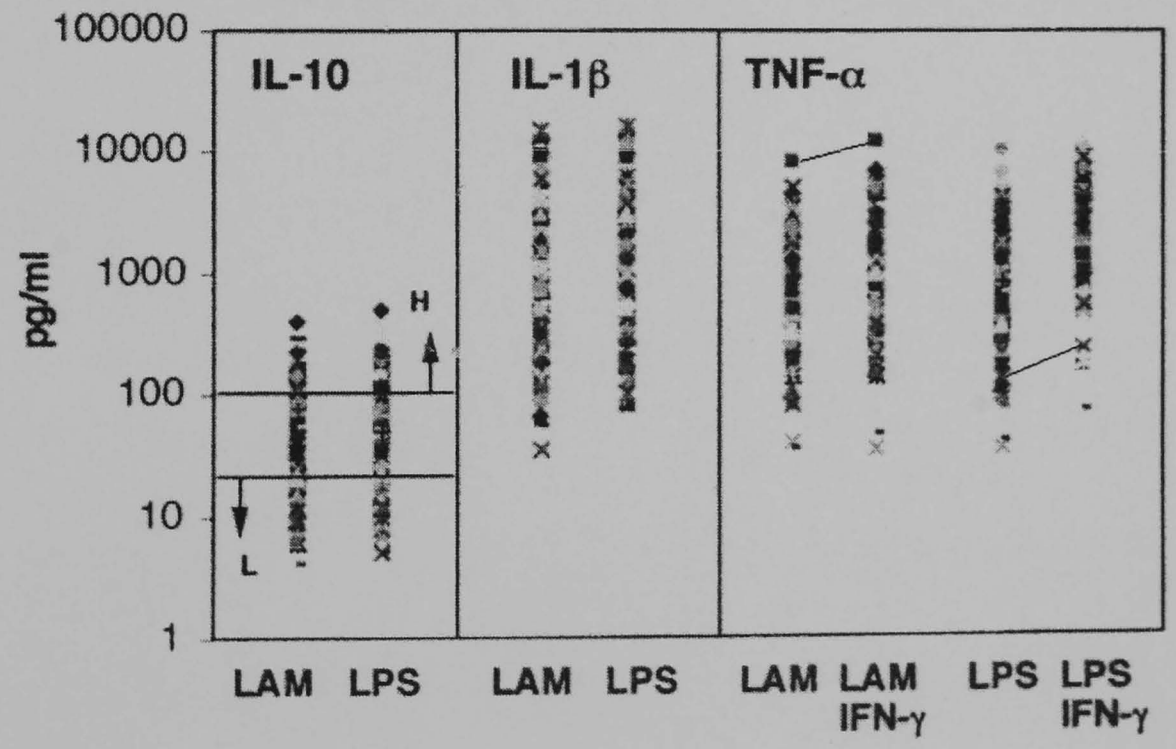


FIG. 3.7 Cytokine production in response to LPS and LAM in healthy individuals

Figure 3.7 shows cytokine levels in response to LPS and LAM stimulation in healthy Gambian individuals. The first column represents IL-10 levels followed by IL-1β levels and lastly TNF-α levels. Abitrary "cut offs" of top 30% (H) and bottom 30 % (L) were set for high and low responder phenotypes respectively. For IL-1β there appears to be a binomial distribution and a natural cut off was possible. Figure 3.7a represents the cytokine measurements and figure 3.7b represents the logarithm transformed data.

As previously shown, the pre-incubating of whole blood with IFN-γ enhanced cytokine responses to LPS and LAM when compared to responses in the absence of IFN-γ priming. However, the mean TNF up-regulation of responses to IFN-γ priming did not differ significantly for the two stimulants being 2.11 for LAM and 2.62 for LPS (Table 3.8). Compared to TNF a lower index of up regulation was observed for IL-1β induced by both LAM (1.44) and LPS (1.88, Table 3.8). LPS was shown to induce higher levels of both cytokines. Pre or co incubation of whole blood with rIFN-γ did not up-regulate the production of IL-10.

Table 3.7 Mean cytokine values in healthy population

CYTOKINES	Mean±S.EM (range) pg/ml			
	LAM	LAM & IFN-γ	LPS	LPS & IFN-γ
TNF	1050 ± 92.0 (35-8100) n=150	1749 ± 141 (35-12345) n=150	911 ± 67 (25-10040) n=270	1758 ± 104 (30-10515) n=275
IL-1β	1749 ± 245 (35-15030) n=144	1846 ± 217 (40-12395) n=144	1405± 142 (10-16265) n=270	1666 ± 155 (10-14310) n=269
IL-10	66 ± 7 (4-386) n=149	53± 4 (3-372) n=149	55 ± 6 (0-707) n=279	38 ± 4 (2-699) n=279

Table 3.7 gives mean and standard error of mean of cytokine released using the different stimulants LAM, and LPS in the presence or absence of IFN-γ. The figures in parentheses are the minimum and maximum observed levels of the corresponding cytokines. n represents the number of individuals studied per stimulant. Cytokine measurements are in picograms per ml.

Table 3.8 Index of IFN-γ upregulation in healthy population

CYTOKINES	Index of IFN-γ upregulation	
	LAM	LPS
TNF	2.11	2.62
IL-1β	1.44	1.88

Table 3.8 shows the index of IFN γ up-regulation that is calculated by expressing TNF production following IFN-γ priming and either LAM or LPS stimulation as a ratio of stimulation with either LAM or LPS alone. This was done for each individual and each cytokine measured. IFN-γ did not up-regulate IL-10 production therefore index of IFN-γ up -regulation of IL-10 was not included in the table.

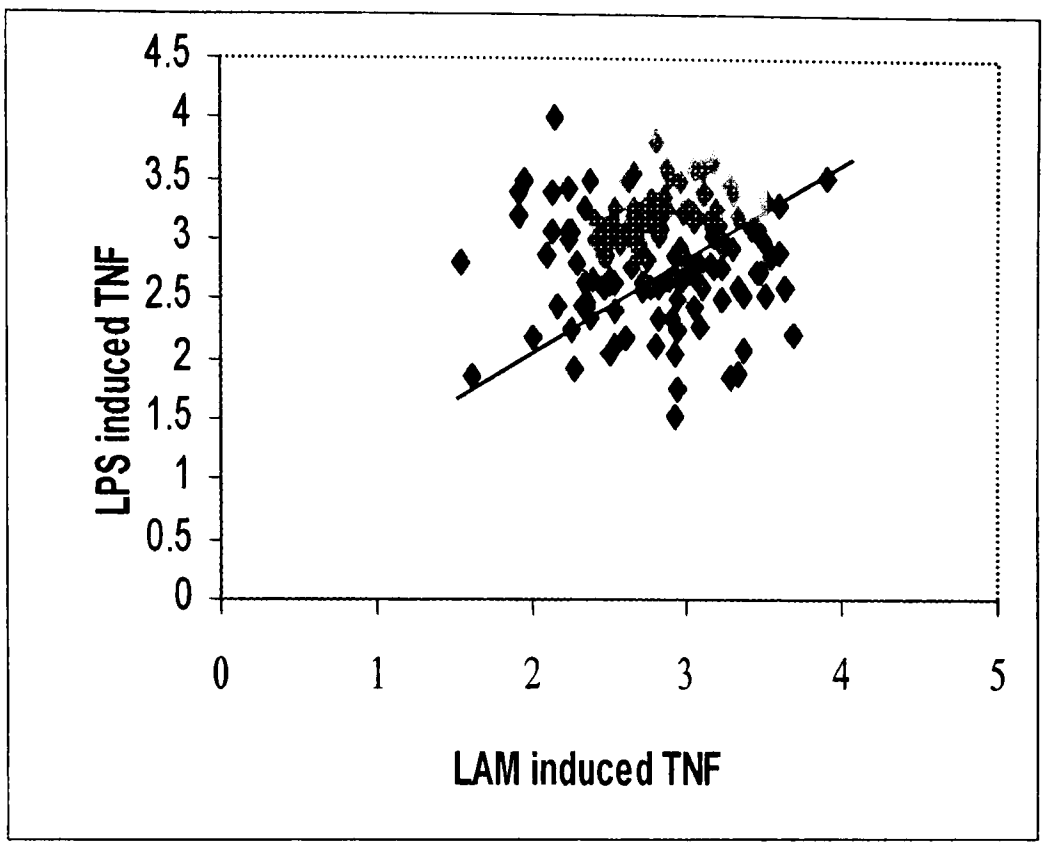


FIG. 3.8.1i TNF induced by LPS versus LAM

TNF induced in response to LPS and TNF induced in response to LAM.

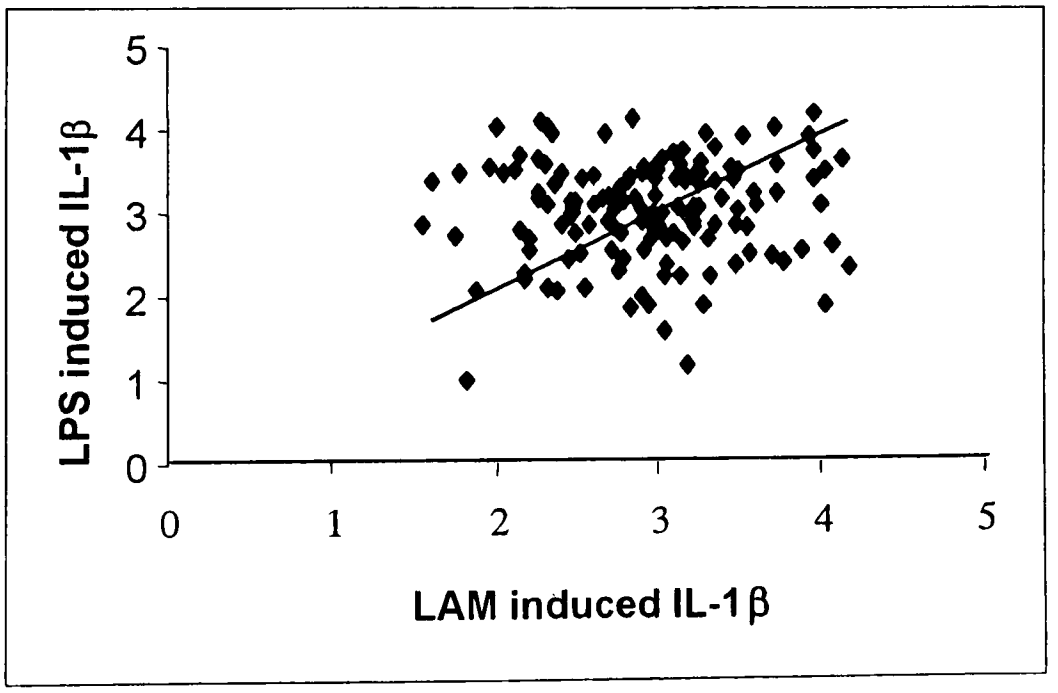


FIG. 3.8.1ii IL-1β induced by LPS versus LAM.

IL-1β induced in response to LPS and IL-1β induced in response to LAM.

FIG. 3.8.1 Positive correlation of TNF and IL-1β induction by LPS or LAM.

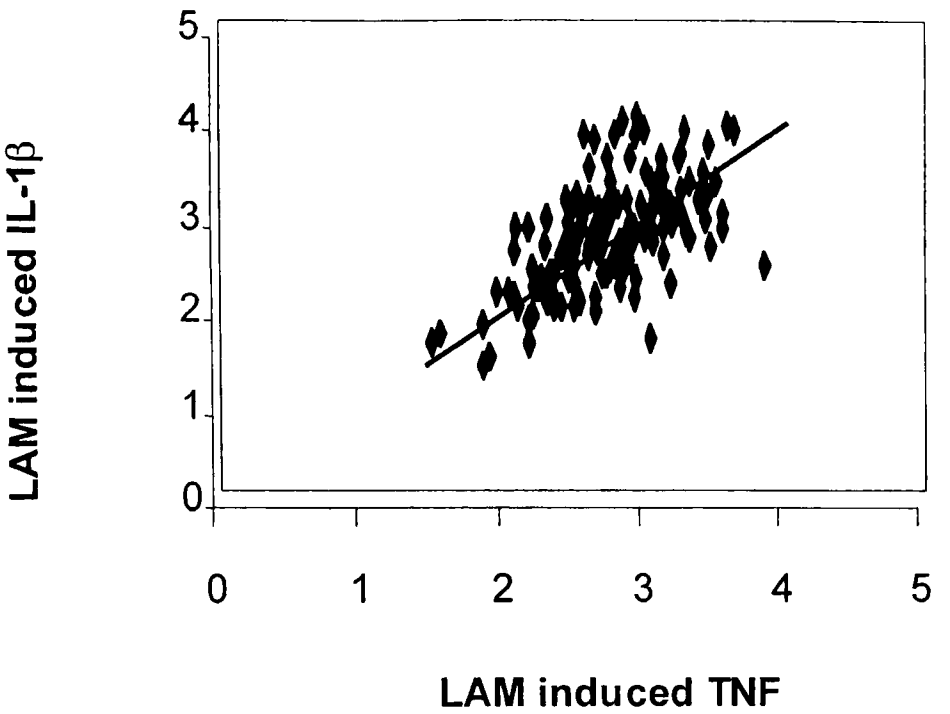


FIG.3.8.2i LAM induced TNF versus IL-1β

The y axis shows IL-1β produced in response to LAM and the x axis shows TNF produced in response to LAM.

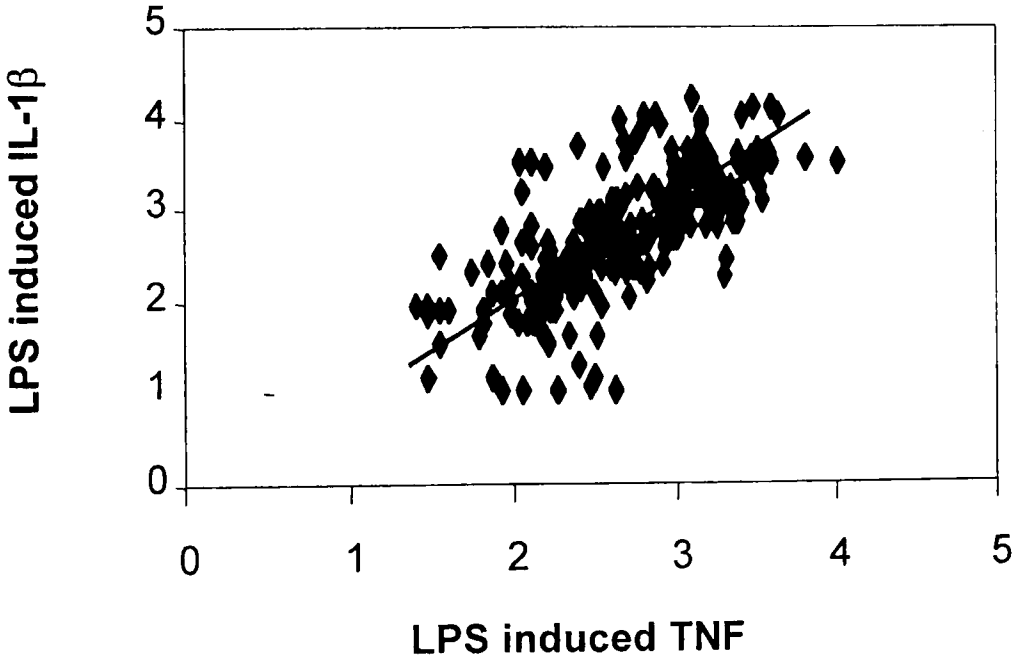


FIG.3.8.2ii LPS induced TNF-α versus IL-1β.

The y axis shows IL-1β produced in response to LPS and x axis shows TNF produced in response to LPS

FIG. 3.8.2 Positive correlation of TNF and IL-1β induction by LPS and LAM.

Table3.9 Correlation of cytokine induction by LAM or LPS in healthy population

Stimulants	Cytokines	Correlation	Observations	p-values
LAM vs. LPS	IL-1β	0.70 (0.68)	137	0.00001
	IL-10	0.74 (0.76)	148	0.00001
	TNF	0.77 (0.78)	145	0.00001
LAM	TNF vs. IL-10	0.55 (0.53)	148	0.00001
	TNF vs. IL-1β	0.60 (0.60)	272	0.00001
	IL-1β vs IL-10	0.42 (0.41)	143	0.00001
LPS	TNF vs. IL-10	0.55 (0.62)	268	0.00001
	TNF vs. IL-1β	0.69 (0.72)	143	0.00001
	IL-1β vs IL-10	0.53 (0.55)	268	0.00001

Data was logarithm transformed and the Spearman's and pairwise correlation were carried out. Stimulants LAM versus LPS represent the correlation of LPS and LAM induced cytokine. LAM induced cytokine values are plotted against LPS for each cytokine (see figure 3.8.1). The Spearman's correlation and pair wise correlation were obtained. Figures in parenthesis are the Spearman's correlation. A positive correlation between 0 and 1 indicates a positive correlation, the higher the value the more correlated the data are. The Spearman's correlation was usually higher than the normal correlation for LPS induced responses whereas the reverse was the case for LAM induced responses. Correlation between induced cytokines by either of the stimulants (LAM or LPS) was ascertained. Highly positive correlation was observed for LAM versus LPS induction of all cytokines (TNF, IL-1β and IL-10 (0.70, 0.74, and 0.77 respectively)). Also positive correlation was observed between cytokines by either of the stimulants. LAM-induced IL-1β versus IL-10 was the least well correlated (0.42). LPS-induced cytokine responses were the most correlated with the highest correlated being TNF versus IL-1β (0.69).

3.4 Discussion

In summary, optimal conditions for TNF, IL-1β and IL-10 release were ascertained in the Gambian population. Parameters for optimal cytokine release using the whole blood assay were necessary since this assay formed the basis for further studies on the genetic regulation of immune responses. Also, it was important to determine minimal intra-assay and inter-individual variation. Conditions obtained were used in subsequent whole blood assays. Marked inter-individual variation in response to both LPS and LAM was observed. There was a significant difference ($p=0.02$) in mean IL-10 levels in response to LPS stimulation between recovered TB cases and controls but this was not observed for TNF and IL-1β. Pre-IFN-γ incubation of whole blood enhanced the production of TNF and to a lesser extent IL-1β; IL-10 production was rather suppressed by pre-IFN-γ incubation. Positive correlation was observed for LAM and LPS induction of TNF, IL-1β and IL-10 in addition there was positive correlation for the release of TNF, IL-1β and IL-10 cytokines.

I have used a whole blood system rather than studying PBMC responses because the whole blood cell culture has many advantages, which were documented in section 3.1.

3.4.1 Higher IL-10 levels in recovered TB cases

IL-10 levels in recovered TB cases are higher than controls ($p=0.02$, Table 3.5.2). Baseline cytokine measurements were done to correct for the effect of differential cytokine production in this population by a variety of infectious agents, as a lot of infections are endemic. For example, variants of *P. falciparium* can down regulate T cell proliferative response by preferential induction of IL-10 (Boussiotis et al., 2000).

Mean IL-10 concentrations were lower in controls (319 ± 100 pg/ml) than in recovered cases (527 ± 282 pg/ml). Corroborating this is a study in leprosy where IL-10 responses are also shown to be lower in control subjects than leprosy subjects or the healthy contact

group (Weir *et al.,* 1996). This is not surprising because several experiments show that IL-10 is a potent monocyte deactivator controlling LPS toxicity. The Weir leprosy study enrolled patients with on going disease. My study selected only people who having completed TB treatment regimen had recovered from tuberculosis and had been off treatment for two months. Recovered TB cases were selected because it is known that TB and treatment would mask any innate underlying defects and could temporarily affect the responsiveness to IFN-γ in individuals (Turner *et al.,* 2000).

The aim was to study the innate system that predisposes to TB and if they had an underlying genetic defect it should still be evident. The observed significant difference in IL-10 levels may represent real innate defect in IL-10 responses in TB. Recently, a study on genetic influence on cytokine production in meningitis demonstrated that it is the initial innate anti-inflammatory cytokine profile that contributes to fatal disease outcome (Westendorp *et al.,* 1997). A role for IL-10 *in M tuberculosis* induced programmed cell death (PCD) of alveolar macrophages was demonstrated by increasing PCD with anti-IL-10 antibody treatment and inhibition of PCD by addition of exogenous IL-10. IL-10 is an anti- inflammatory cytokine that has been reported to inhibit TNF, IL-1β, IL-12, IL-8, IL-6, and GM-CSF production, release of free radicals and nitric- oxide and thereby suppress the anti-microbial properties of macrophages against various pathogens. Therefore, IL-10 interferes with very early events of monocyte and neutrophil activation by microbial products. In endotoxemia, TNF appears to promote, but is not essential for, the production of IL-10 by LPS stimulated human monocytes (Marchant *et al.,* 1999b). Also, IL-1β derived from monocytes can stimulate the production of IL-10. It appears that the initial production of these molecules is beneficial and indicates immune competence. Therefore, if increased level of IL-10 may be indicative of fatal disease outcome; it would act earlier at the onset of disease and suppress the actions of the other cytokines. A recent study by

Boussiotis *et al.,* (2000) showed that constitutively produced IL-10 by T cells suppressed immune responses in anergic TB cases. They showed that invitro stimulation of T cells in PPD^+ cases induced IL-10, IFNγ, and proliferation whereas only IL-10 was induced in PPD^- cases. This difference was shown to be as a result of defective hypophosphorylation of TCRζ and defective activation of ZAP-70 and MAPK (Boussiotis *et al.,* 2000). We may not see any significant difference in pro inflammatory cytokine levels between cases and controls as it is assumed that individuals who recover after treatment are able to mount an appreciable amount of cellular immunity to augment the actions of treatment. A study that demonstrated significant levels of TNF in plasma between TB cases and controls used peripheral blood mononuclear (PBMC) cells and this study was in acute TB cases and not in recovered patients (Friedland, 1995).

However, we observed no difference in mean TNF and IL-1β levels between recovered TB cases and controls (Table 3.5.1 & 3.5.2). The explanation for this maybe that pulmonary TB being more of a localised infection blood sampling may not necessarily depict the true cytokine situation and more localised samples such as lung aspirates or broncho-alveolar lavage may be required. Though relevant, it is often not easy to obtain and study alveolar macrophages. If blood samples must be used then pro and anti-inflammatory cytokines produced by other cells such as PNG may be important to measure. Such cells first migrate from blood into tissue sites and participate in early inflammatory response. Measuring molecules that are chemotactic for macrophages such as, the small inducible chemokines may important in TB.

Interesting data emerging from ex-vivo whole blood studies have suggested an important role for IL-8 in TB. A study in Zambian patients with both HIV and tuberculosis showed that higher levels of IL -8 were present in plasma of survivors than those who died, after ex vivo stimulation of whole blood leukocytes. As already mentioned, measurement of

secondary inflammatory cytokines such as IL-8 would be important (Friedland *et al.*, 1995). In addition to whole blood assay and cytokine measurements one could quantify cell surface receptors as this may give an idea of correlation between receptor usage and cytokine levels observed. This may aid in our understanding of mechanisms involved in macrophage function.

However, this study demonstrates inter-individual variations in cytokine responses induced by manLAM and LPS. Priming with IFN-γ before stimulating with either LAM or LPS was shown to potentiate the release of TNF and IL-1β. IL-10 levels were depressed.

3.4.2 Correlations of cytokine responses

Positive correlation was observed for either LAM or LPS induced TNF and IL-1β and also there was positive correlation for TNF and IL-1β induced responses.

Positive correlation observed for LAM and LPS induction of TNF and IL-1β induction suggests that manLAM and LPS may activate macrophages through the same cell surface receptors or post receptor pathway. CD14, a phosphatidylinositol glycan-linked membrane protein best known and characterised as the high affinity receptor for lipopolysaccharides from gram negative bacteria can also bind LAM from *M. tuberculosis* (Pugin *et al.*, 1994). However, Zhang et al (1993) showed that LAM and LPS possibly activate polymorphonuclear granulocytes (PNGs) through a receptor different from CD14. They went on to show that cytokine induction in human monocytoid cell line THP-1 by LAM and LPS was not inhibited by a monoclonal antibody to CD14 suggesting other common receptor for LAM and LPS expressed on macrophages. The toll like receptor (TLR) signalling systems may be important in this mechanism (Kopp and Medzhitov, 1999).

TLRs were first described in the fruit fly *Drosophila melanogaster* where they were involved with dorsoventral pattern formation and later on in the adult fly's life, they are involved with anti-microbial activity (Rock *et al.,* 1998). Human homologues can mediate innate immune recognition by binding to conserved molecular patterns on microorganisms. These receptors are coupled to signal transduction pathways that control expression of a variety of inducible immune-response genes. The cytoplasmic domain of the Toll-family proteins is homologous to the cytoplasmic domain of the IL-1 receptor (IL-1R) family. Five TLRs have been reported in man (TLRs 1, 2, 3, 4, 5). LPS can activate macrophages via TLR 2 (Kirshching *et al.,* 1998,Yang *et al.,* 1998) and TLR4 (Heine *et al.,* 1999, Chow *et al.,* 1999 and Poltorak *et al.,* 1998). ManLAM can activate macrophages via TLR-2 (Means *et al.,* 1999). Means *et al.,* (1999) also showed that viable *M. tuberculosis* bacilli activated both Chinese hamster ovary cells and murine macrophages that over expressed either TLR2 or TLR4. This suggests that in vivo, *M. tuberculosis* can utilise both TLRs for signalling.

Since TNF and IL-1β are pro inflammatory cytokines, induced by the same agents, activate the same kinases and induce similar effects such as fever, it is not surprising that positive correlation was observed in their induction by LAM or LPS. Both the TNF and IL-1β genes have a 3' untranslated sequence that is responsive to LPS and which affects mRNA stability (Caput *et al.,* 1986). In addition, similar pathways are required for the induction of synthesis of these cytokines. This data is in contrast with another study that was unable to demonstrate any correlation for the induction of IL-1β and TNF in mice (Nettea *et al.,* 1998). Nettea *et al.,* (1998) showed that LPS induced production of TNF and IL-1β was differentially regulated at the receptor level. It was clearly demonstrated that TNF production was essentially CD14 dependent, both in the presence or absence of plasma. However, LPS induced IL-1β production was mediated by CD14 dependent

mechanisms in the presence of plasma, while in its absence both CD14–dependent and CD14-independent pathways are involved. A report such as this is awaited for LAM.

In addition, there was marked heterogeneity of cytokine produced with or without IFN-γ priming of whole blood. Priming of IFN-γ of whole blood prior to LPS or LAM incubation, showed that individuals could further be classified according to their ability to up regulate monocyte function as judged by levels of TNF or IL-1β produced. A study by deWitt *et al.,* (1996), demonstrated that IFNγ modulates the expression of several cytokines by human monocytes at the transcriptional level. Indeed, it was shown that priming of human monocytes with IFN-γ resulted in the down regulation of *c-fos* and *c-jun* mRNA and AP-1 DNA binding capacity in response to stimulation with LPS compared to the effects of LPS alone. Interestingly, an increased NF-Kβ expression was noticed in IFN-γ pre-treated monocytes (de Witt *et al.,* 1996). This study observed an increase in protein levels of pro inflammatory cytokines in response to IFN-γ pre-treatment of whole blood followed by LPS stimulation. All together, this suggests that the IFN-γ pathway may therefore be an important contributory factor to the variations observed and to inflammatory processes of macrophage activation.

3.4.3. Molecular basis of heterogeneity of cytokine responses.

The inter-individual variability observed may result from differences in host genetic constitution. It is known that changes in DNA sequence of a gene can cause alterations in the function of the gene. Single change in just one base can lead to physiological abnormal conditions. A good example is sickle cell anaemia where a CTT to CAT leads to an amino acid substitution that alters the way the haemoglobin molecule folds, thereby distorting the shape of the red blood cell. Control of gene activity could therefore be at the point of transcriptional activation of the gene, stability of mRNA, translational modification, intracellular stability of protein, its transport and ultimately at the point of

export of the gene product. For example, polymorphisms in or around transcription factor binding sites within promoter regions of a gene may affect the rate transcription of the gene. A good example of this the OCT-1 binding site the promoter region of the TNF gene (Knight *et al.,* 1999b). A polymorphism at this site influences binding of OCT-1 and is also associated with susceptibility to malaria. Whilst sickle cell anaemia is an excellent illustration of the relationship between a single point mutation in DNA and loss of protein function, most changes in DNA sequence may be less dramatic.

Many pathways require molecules encoded by different genes. A process such macrophage activation requires several molecules and therefore many genes. Alterations in DNA sequence of genes that are involved in macrophage activation would therefore be important in understanding the genetic basis of functional heterogeneity.

In addition to genetic factors, other factors which may be responsible for observed inter individual variation in cytokine responses observed may be explained by differential production of soluble inhibitory substances in plasma of individuals. Soluble forms of the cytokine receptors are thought to bind cytokines and cause prolonged bioactive half-life of cytokine within circulation and inhibit binding of cytokine to membrane associated cell surface cytokine receptor for subsequent macrophage activation pathway. In support of this is the recent report by McGuire *et al.,* (1998), which demonstrated varied amounts of sTNF R often in ng/ml concentrations in both malarial and healthy individuals.

In conclusion, the demonstrations of marked inter-individual variations in the ability to produce these cytokines maybe as result of individual innate genetic variability in macrophage function.

Polymorphism at candidate gene loci were therefore studied to determine if these genes regulate microbial induced macrophage responses.

CHAPTER 4

NOVEL PROMOTER REGION POLYMORPHISMS IN THE *IFNG* AND *IFNGR1* GENES

4.1. Introduction

The interferon –γ pathway has been implicated in the immune response to virtually all infectious agents including fungi, viruses, parasites and bacteria. IFN-γ is produced by NK and T cells and has a modulating role in almost all phases of the immune response (Trincheri and Perussia, 1985). This includes stimulation of bactericidal activity of phagocytes (Momburg *et al.,* 1986), the stimulation of antigen presentation through class I and class II MHC molecules, the orchestration of leukocyte-endothelium interactions, cell proliferation and apoptosis. IFN-γ is associated with the development of Th1 like cell-mediated immunity, required for protection in mycobacterial diseases (Flynn *et al.,* 1993, Newport *et al.,* 1996).

Figure 1.3 shows a schematic representation of the mechanism of signal transduction for the IFN-γ pathway. Upon binding of IFN-γ, a homodimeric ligand to the receptor α chain (IFN-γR1), there is rapid dimerisation of the receptor α chain, and a site recognised by the extracellular domain of the receptor β subunit (IFN-γR2) is formed (Bach *et al.,* 1997). There is juxtapositioning of the intracellular domains of the two-receptor chains resulting in the transactivation of the enzyme Janus kinases JAK1 and JAK2. There is phosphorylation of the functional tyrosine residue at position 440 on the receptor α subunit, forming a pair of Stat1 docking sites on the ligated receptor. Two Stat1 molecules associate with the paired docking sites and are brought into close proximity with receptor associated activated Janus kinase (JAK) enzymes and are activated by phosphorylation of Stat 1 tyrosine residue at position 701. Tyrosine phosphorylated Stat1 molecules dissociate from their receptor and form homodimeric complexes. The activated Stat1 complex is phosphorylated. Activated Stat1 translocates from the cytoplasm into the nucleus and after binding to a specific

sequence in the promoter regions of immediate early IFN-γ inducible genes, termed gamma interferon activation site (GAS), and effects gene transcription in the presence of RNA polymerase. GAS is a 9 nucleotide site with a consensus sequence of TTNCNNNAA (Figure 4.13, Bach *et al.*, 1997). This sequence is present in the promoter of all IFN-γ inducible genes.

Given the central role of the IFN- γ pathway the immune response to mycobacterial infection, it was then determined to test the hypothesis that genetic variation in components of the pathway influences the magnitude of the immune response and ascertain its effect in determining susceptibility to disease. This in turn requires identification of genetic variants (polymorphisms) within the genes of the pathways to study in relation to either the immune response or disease susceptibility. Since the pathophysiology of many disease's (both infectious and autoimmune) is believed to be due to aberrant T helper type 1 (Th1) responses, and there is epidemiological evidence that host genetic factors influence susceptibility to these diseases, many groups have searched for variation in *IFNG*. Interestingly, relatively few polymorphisms have been described. The gene for human IFN-γ is located at chromosome 12q24.1 (Gray & Goeddel, 1982). One variable length CA repeat polymorphisms have been described (Ruiz-Linares A, 1993). Homozygotes for allele 2 of the CA repeat located within intron 1 of *IFNG*, have been shown to produce more IFN-γ than other alleles (Pravica *et al.*, 1999). This allele has also been shown to be associated with development of fibrosis in lung allograft (Awad *et al.*, 1999) while allele 3 appears to be associated with type 1 diabetes (Jahromi *et al.*, 2000).

However, few SNPs have been reported either in the coding or non-coding regions, to the extent that it has been suggested that IFN-γ plays such a critical role in host

defence that variation within the gene has not been tolerated. A promoter mutation at –333 bp of the initiation codon was reported (Giedraitis *et al.*, 1999), but only observed in 2 individuals (heterozygotes) with multiple sclerosis out of 300 samples typed. Bream et al (2000) sequenced the *IFNG* non-coding regions in 100 individuals and found no promoter polymorphisms. They did however identify a single nucleotide insertion in intron 1, three SNP's in intron 3 and a SNP in the 3' untranslated region.

IFN-γ exerts its pleiotrophic effects via its species-specific receptor, IFNγR. IFN-γR is expressed on all nucleated cells. The receptor consists of two chains, the alpha chain (IFNγR1) which binds ligand and is encoded on chromosome 6q23-24, the beta chain (IFNγR2) is responsible for signal transduction and is encoded on chromosome 21. Mutations in *IFNGR1* and *IFNGR2* have been shown to confer susceptibility to severe infection with nontuberculous mycobacteria (Newport et al 1996, Jouanguy *et al.*, 1996, Dorman and Holland, 1998). However, these mutations are extremely rare at the population level and have only been found in the families in which the disease is segregating. There were only two reported polymorphisms for *IFNGR1*, one intron six CA repeat (FA1, Altare *et al.*, 1998b) and one reported *TaqI* RFLP (Hauptschein *et al.*, 1992). The intron six repeat of *IFNGR1* showed marginal association with total serum IgE levels in the British population (Gao *et al.*, 1999). Recently a new polymorphism at the COOH terminal of the signal peptide of *IFNGR1*; a substitution of valine for methionine (Val14Met) at amino acid position 14 was shown to be associated with systemic lupus erythematosus (SLE). This polymorphism alters receptor function (Tanaka *et al.*, 1999). A coding variant of the *IFNGR2*; glycine to arginine substitution at amino acid position 64 was also associated with total serum IgE in the British population (Gao *et al.*, 1999).

In chapter 3 I presented data demonstrating that there is a marked inter-individual variation in immune responses to LPS and LAM. This occurs both with and without IFN-γ priming. There may be differences in the level of activation of monocyte function in response to IFN-γ mediated by variation in receptor function. In order to investigate this hypothesis further, it was necessary to identify polymorphisms in *IFNG* and *IFNGR1* present in the Gambian population. Promoter regions are important because they contain regulatory sequences of DNA at which RNA polymerase binds to initiate transcription. Variants that exact more subtle effects on the function of gene product may be important in susceptibility to TB in an adult population.

The inter-individual variations observed in macrophage activation could be directly or indirectly due to individual responsiveness to IFNγ, which may be as a reflection the effect of IFN-γ binding to its receptor. Those lethal, inactivating and rare mutations described in chapter 1.2, which are responsible for Mendelian disorders, give important evidence that IFN-γ activation of macrophage is important for containment of mycobacteria. However, these were not typed during the course of this study because they are unlikely to be relevant to disease susceptibility at the population level. Also for IFN-γ there were only two reported polymorphisms. New polymorphisms in the promoter regions of both IFN-γ and the IFN-γ receptor had to be identified.

4.2. Methods

Specific promoter regions of the *IFNG* and *IFNGR1* genes were sequenced using the thermal cycle sequencing method.

4.2.1. Sequencing promoter regions of IFN-γ

Genomic DNA extracted as stated in chapter 2 by standard phenol/chloroform method was used as template for amplification. Samples from 36 genetically independent Gambian TB cases were sequenced.

Thermal cycle sequencing was carried out in two stages. An initial amplification of the 1.6kb fragment upstream of the transcription start site was carried out. Subsequently, the region was divided into 3 fragments of 569bp, 555bp and 631bp for sequencing. Primers to amplify this region were designed by Dr. Jeremy Hull in Professor Kwiatkowski's laboratory in Oxford as listed below from published sequence. The M13 Big dye primer forward sequencing kit was used and polymorphisms and ambiguities were confirmed using M13 Big dye primer reverse kit.

Table 4.1. Details of primer sequence for the human *IFNGR1*-γ promoter region

Primer name	Sequence	Position relative to initiation codon
Primary primers		
F2	5'ACTCACAATCATATAGCTAG3'	-1486-1466bp
R1	5'CTGATCAGCTTGATACAAGA3'	+46-+66bp
Second round primers	M13 tailed	
F3	5'TAACCAACTCTGATGAAGG3'	-541-485bp
R2	5'CAGTTAAGTCCTTTGGACC3'	-985-967bp
R4	5'CTTTCCTTGCTTTCTGGTCATT3	-451-430bp
F5	5'TTCAAGCCATTCTCCTGCC3'	-985-967bp
F4	5'ACTCACAATCATATAGCTAG3'	-1485-1466bp
R3	5'GGCTAGGCTGGTCTCAAACT3'	-875-856bp

F = forward primer

R = reverse primer

Primary PCR reaction mixes were in 15µl volumes made up of 50ng/ml of primers, 100ng/ul of DNA, Bioline 10X Opti Buffer, 2.5mM Mg Cl_2, 5mM dNTPs, 0.1U Bio Xact Taq DNA polymerase and water. Confirmation of product size was carried out on a 1.5- % agarose gel with ethidium bromide staining and visualised by UV light. Secondary PCR reaction mixes was in 15µl volume made up of 50 ng/ml of primers, 1

μl of primary PCR product, 0.5 mM dNTPs, 2.5 mM $MgCl_2$ 10X ampliTaq Gold Buffer and 0.075U of ampliTaq Gold.

The thermal cycle conditions for primary PCR were an initial denaturation for 94°C for 2 minutes, followed by 9 cycles of 94°C denaturation for 15 seconds, annealing at 58°C for 30 seconds, then elongation at 68°C for 2 minutes. This was followed by additional 19 cycles of 94°C denaturation for 15 seconds, annealing at 58°C for 30 seconds and elongation at 68°C for 2 minutes. An additional 20 seconds was added per cycle and there was final elongation at 68°C for 5 minutes.

Thermal cycle conditions for secondary PCR were an initial 94°C denaturation for 10 minutes, followed by 30 cycles of 94°C denaturation for 30 seconds, annealing at 54°C for 30 seconds and elongation at 72°C for 2 minutes. Final elongation was at 72°C for 5 minutes.

PCR product was electrophoresed on an ABI PRISM® 377 DNA sequencer as described in chapter 2.

4.2.2. Sequencing promoter regions of *IFNGR1*

Genomic DNA extracted as stated in chapter 2 by standard phenol chloroform method was used as template for amplification. Samples from 36 genetically independent Gambian TB cases were sequenced using thermal cycle sequencing.

This was carried out in two stages. An initial amplification of the –964bp fragment upstream of the transcription start site was done. Subsequently, a second round sequencing of 594 bps relative to the initiation codon was done. Primers to amplify

this region were designed by Dr. Melanie Newport in The Gambia and secondary primer set was by Jeremy Hull in Professor Kwiatkowski's laboratory in Oxford as listed.

Table 4.2. Details of primer sequence for human *IFNGR1* promoter region

Primer name	**Sequence**	**Position relative to initiation codon**
Primary PCR primers.		
IFNGR1	5'GAGTGCCAAGTAAAGATGTCAG3'	-961 to –941bp
IFNGR4	5'AGATCCGCGGTGCCACTCA3'	-+51 to +71bp
Second round primers	M13 tailed	
GAR F2	5'AGTAGTTCTTGGTCAAGCCG	-574 to-594bp
GAR R2	5'CTCTCCTCTTTCTCCTACCC	+5 to +24bp

The M13 Big dye primer forward sequencing kit was used and polymorphisms and ambiguities were confirmed using M13 Big dye primer reverse kit.

Primary PCR was carried out in 100µl volumes consisting of 2µl of 100ng/ml template DNA, 100 mM Tris, 500 mM KCl, 1.5Mm $MgCl_2$ (pH 8.3), 20mM dNTPs 33 ng/ml primer, 0.1unit ampliTaq DNA polymerase and 74.4µls of water, using a Cyclogene thermal cycler. Confirmation of product size was carried out on a 1.5- % agarose gel stained with ethidium bromide and visualised by UV light.

After initial amplification of samples, subsequent purification of PCR product was done using the Promega miniprep purification kit following manufacturers instructions.

Secondary PCR was done in a total reaction volume of 15µl made of 50ng/ml of primers, 1ul of primary PCR product, 0.5mM dNTPs, 2.5mM $MgCl_2$, 10X ampliTaq Gold Buffer and 0.075U of ampliTaq. Gold.

The thermal cycle conditions for primary PCR were an initial denaturation for 95°C for 2 minutes, followed by 30 cycles of 95°C denaturation for 1 minute, annealing at 50 for 30 seconds, then elongation at 72°C for 1.30 minutes followed by a final elongation of 72°C for 7 minutes.

Thermal cycle conditions for secondary PCR were an initial 94 ^{0}C denaturation for 10minutes, followed by 30 cycles of 94^{0}C denaturation for 30 seconds annealing at 54^{0}C for 30 seconds, elongation at 72^{0}C for 2minutes and a final elongation at 72^{0}C for 5 minutes.

PCR product was electrophoresed on a ABI PRISM® 377 DNA sequencer as stated in chapter 2

4.3 Results

Generally three patterns are expected when novel polymorphisms/mutations are detected: the homozygote normal which should be the commoner or wild type mutation, the homozygote mutant which should be the less common type, and the heterozygote where the two chromosomes homologue carry different variants of the mutation. Using the ABI 377 sequencer, the heterozygous individual has two detectable peaks at the point of mutation whereas only one peak was observed for the

homozygous mutant (Figure 4.1). The software programme allows the detection of a four-colour trace. Point mutations of the two homozygotes and heterozygote were clearly detectable on the traces (Figure. 4.1). Base substitution was either transition or transversion change. Also a deletion mutation was identified. Homozygous normal, homozygous deleted and heterozygote individuals were also detected (Figure 4.1). The heterozygous individuals were recognisable because the sequence traces beyond the mutation became unreadable due to overlapping sequences from the two chromosome homologues. Five simple point mutations were detected within the *IFNG* gene promoter region while one double deletion, one simple point polymorphism and three simple point mutations were detected within the promoter region of the *IFNGR1* gene in the samples analysed (Figure. 4.14). In all 10 new mutations were identified. The electropherographs showing the different polymorphisms can be seen below through Figure. 4.2 to.4.6 and Figure 4.8 to 4.12.

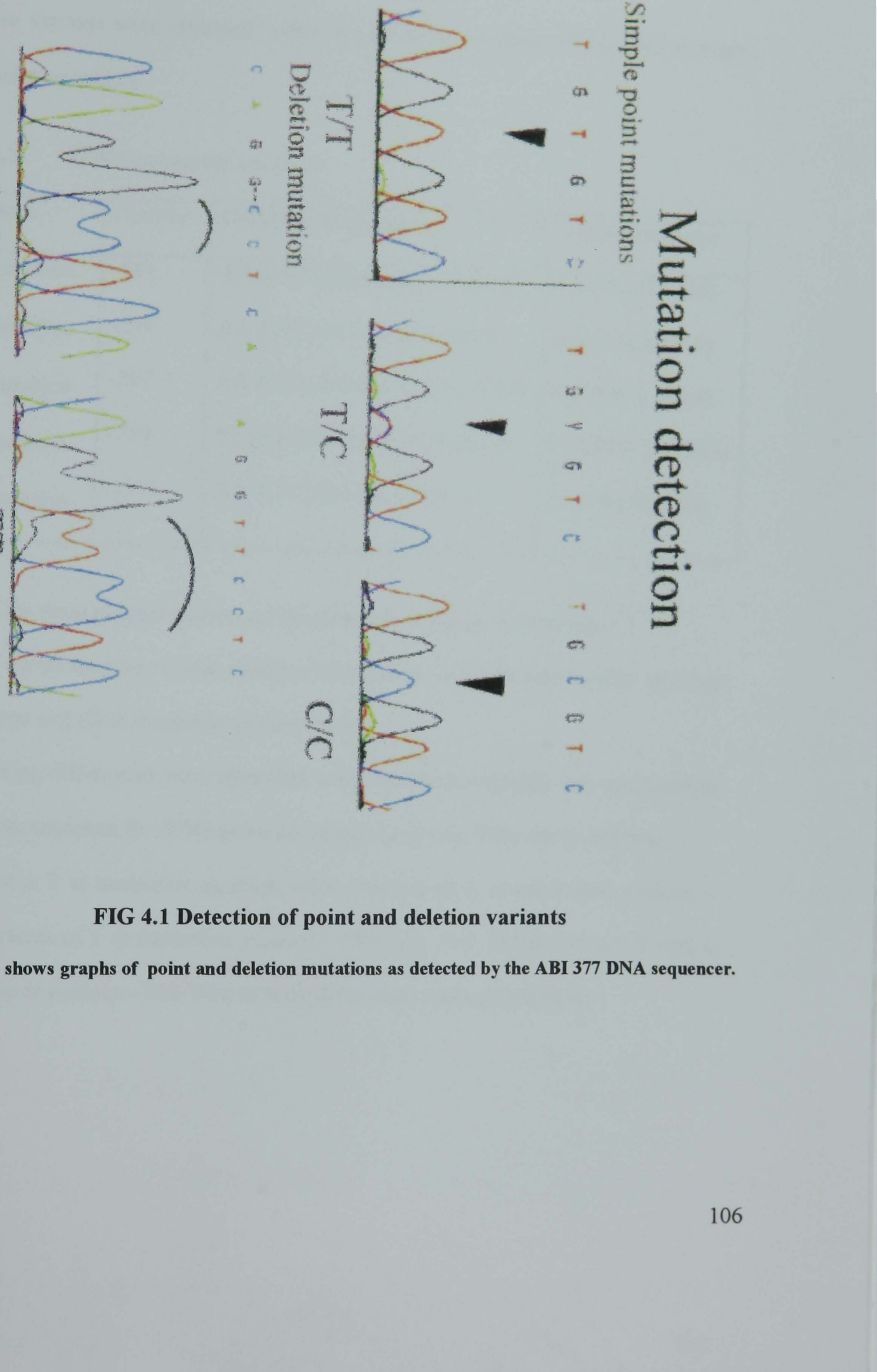


FIG 4.1 Detection of point and deletion variants

Figure 4.1 shows graphs of point and deletion mutations as detected by the ABI 377 DNA sequencer.

4.3.1. Sequence variation in the *IFNG* promoter region

Five new variants were observed within the promoter of human IFN-γ. All five are single point mutations.

Table 4.3. *IFNG* promoter variants

Substitution	Position	Genotype frequency	Allele Frequency
A→G transition	-151	AA (0.97) GG (0) G/A (0.03)	A (0.99) G (0.01)
A→G transition	-234	AA (0.97) GG (0) G/A (0.03)	A (0.99) G (0.01)
G→A transition	-297	AA (0.97) GG (0.33) G/A (0.03)	G (0.99) A (0.01)
C→T transition	-790	TT (0.03) CC (0.86) C/T (0.11)	C (0.915) T (0.085)
A→G transition	-1371	AA (0.95) GG (0) A/G (0.05)	A (0.98) G (0.02)

Evidence for these polymorphisms are documented in Figure 4.3 through 4.7.

Table 4.3 shows the type of base changes followed by the nucleotide position and then the genotype and allele frequency in parentheses.

The following differences were identified when Gambian sequence was compared to the Genbank sequence for *IFNG* promoter region sequence. They are as follows.

Insertion of a T at nucleotide position –237, deletion of A at nucleotide position –1319. Insertions of T at nucleotide positions –908 and –561. Substitution of T with a C at nucleotide position –764. Non of these differences were polymorphic

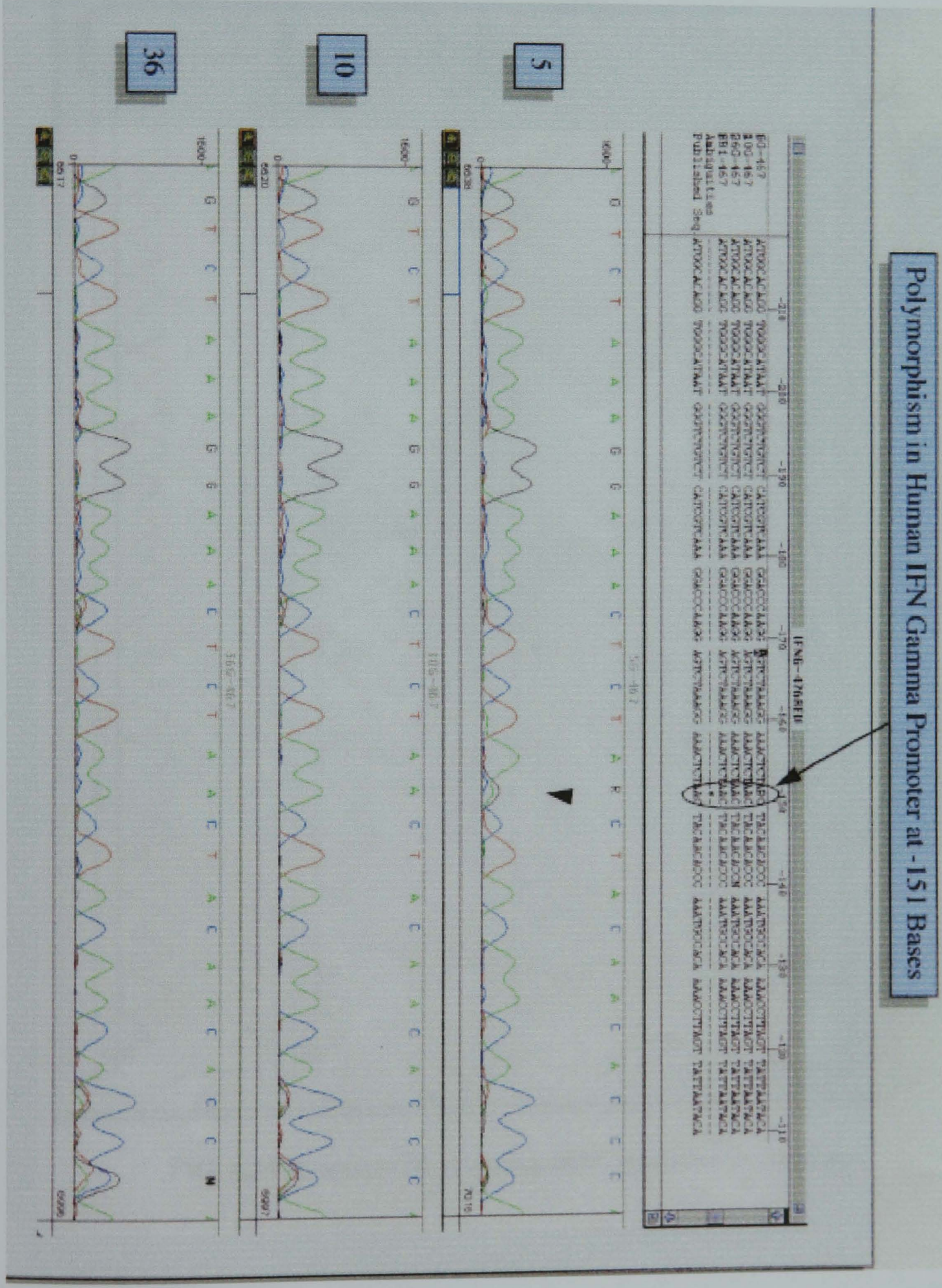


Figure 4.2 Polymorphism of human *IFNG* promoter at -151 bases.

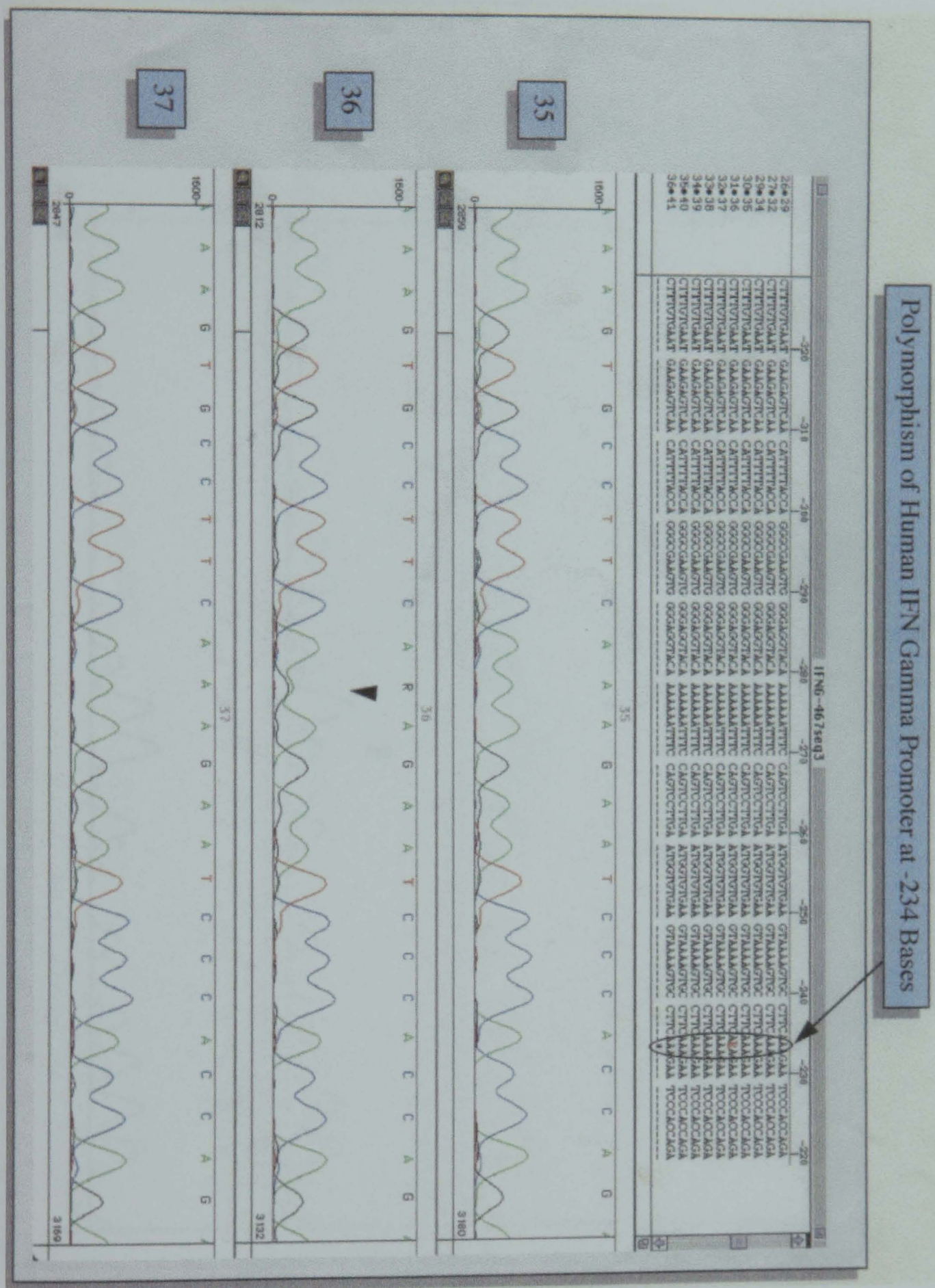


FIG. 4.3 Polymorphism of human *IFNG* promoter at -234 bases

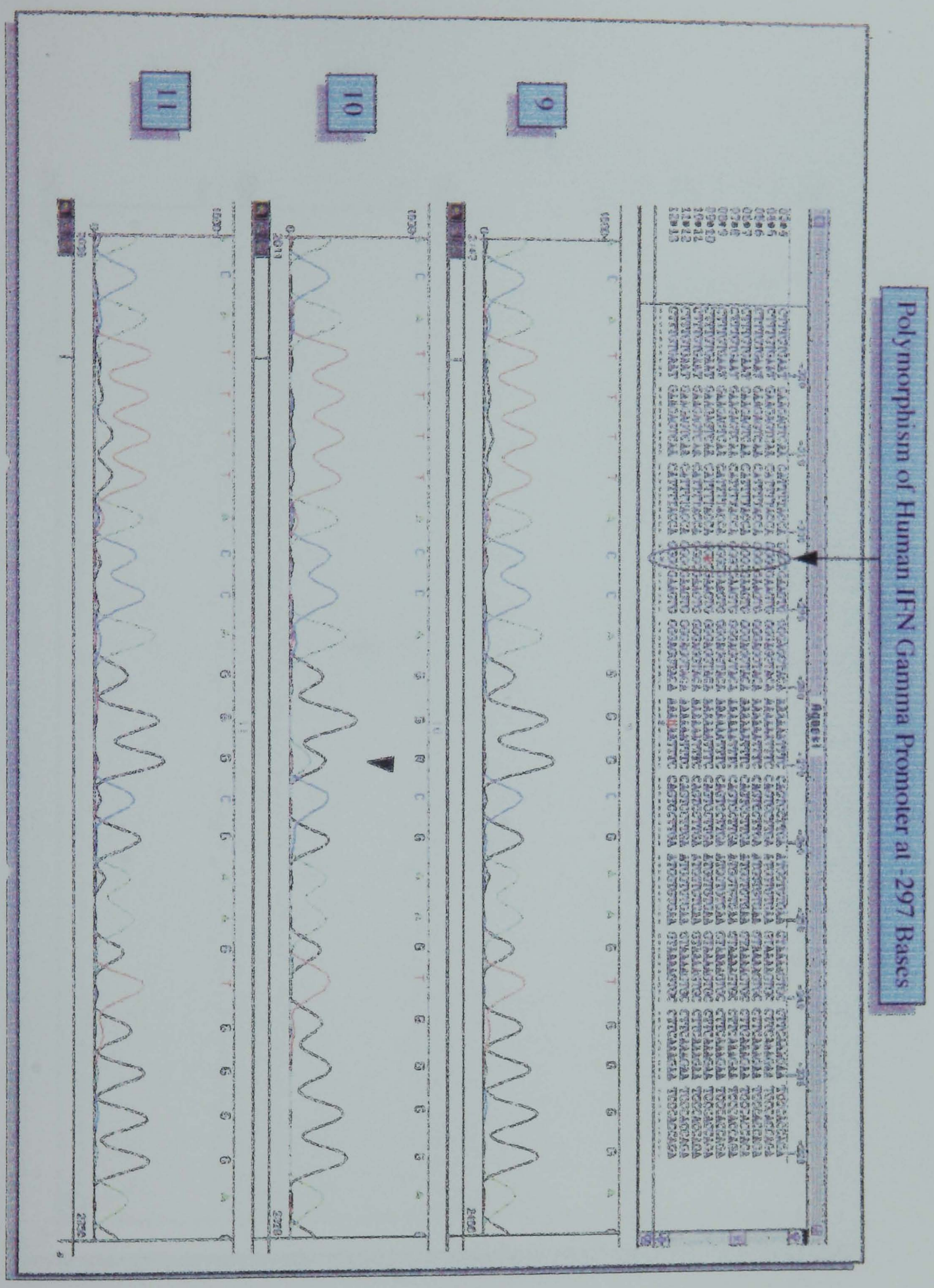


FIG 4.4 Polymorphism of human *IFNG* promoter at -297 bases

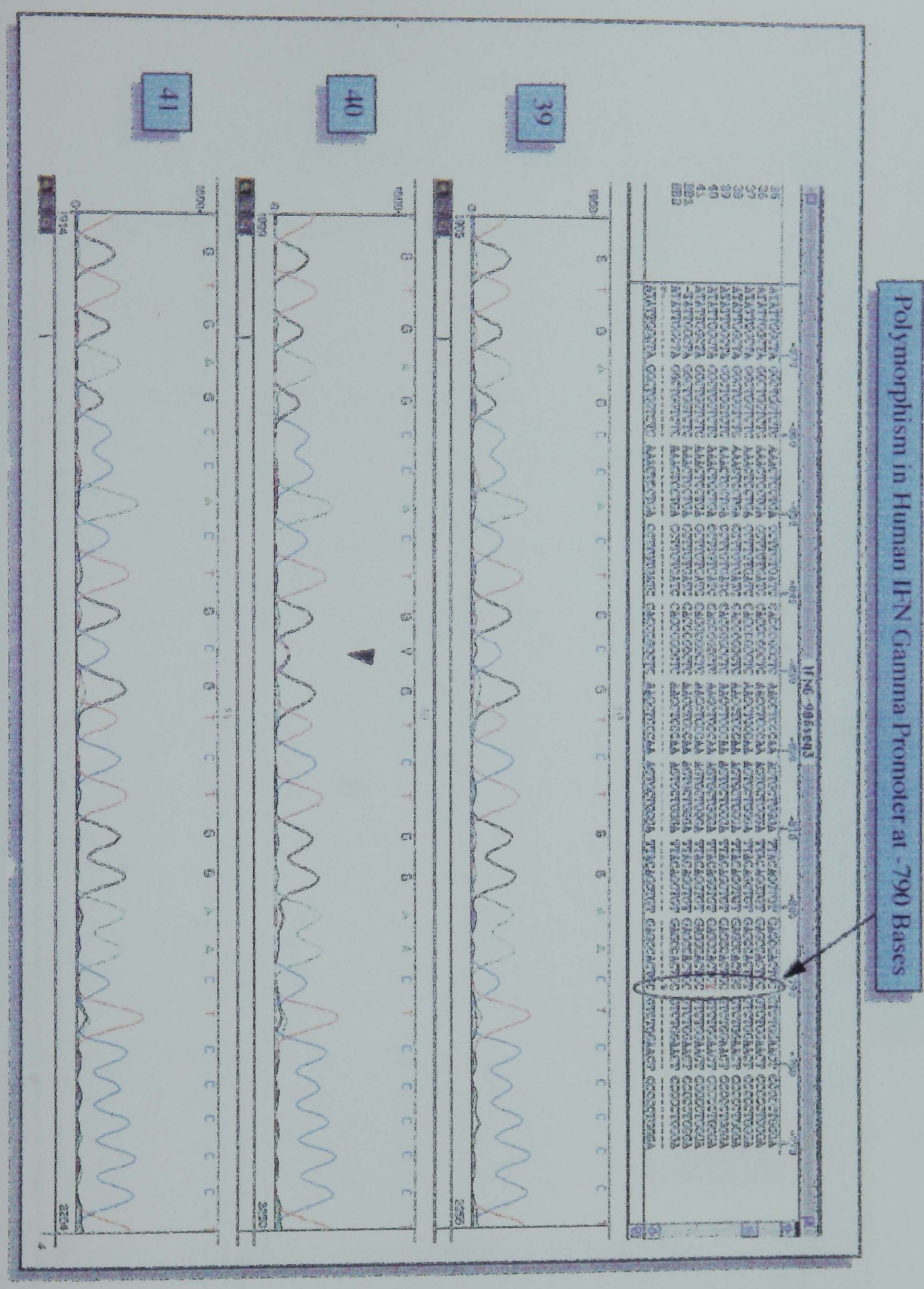


FIG 4.5 Polymorphism of human *IFNG* promoter at -790 bases

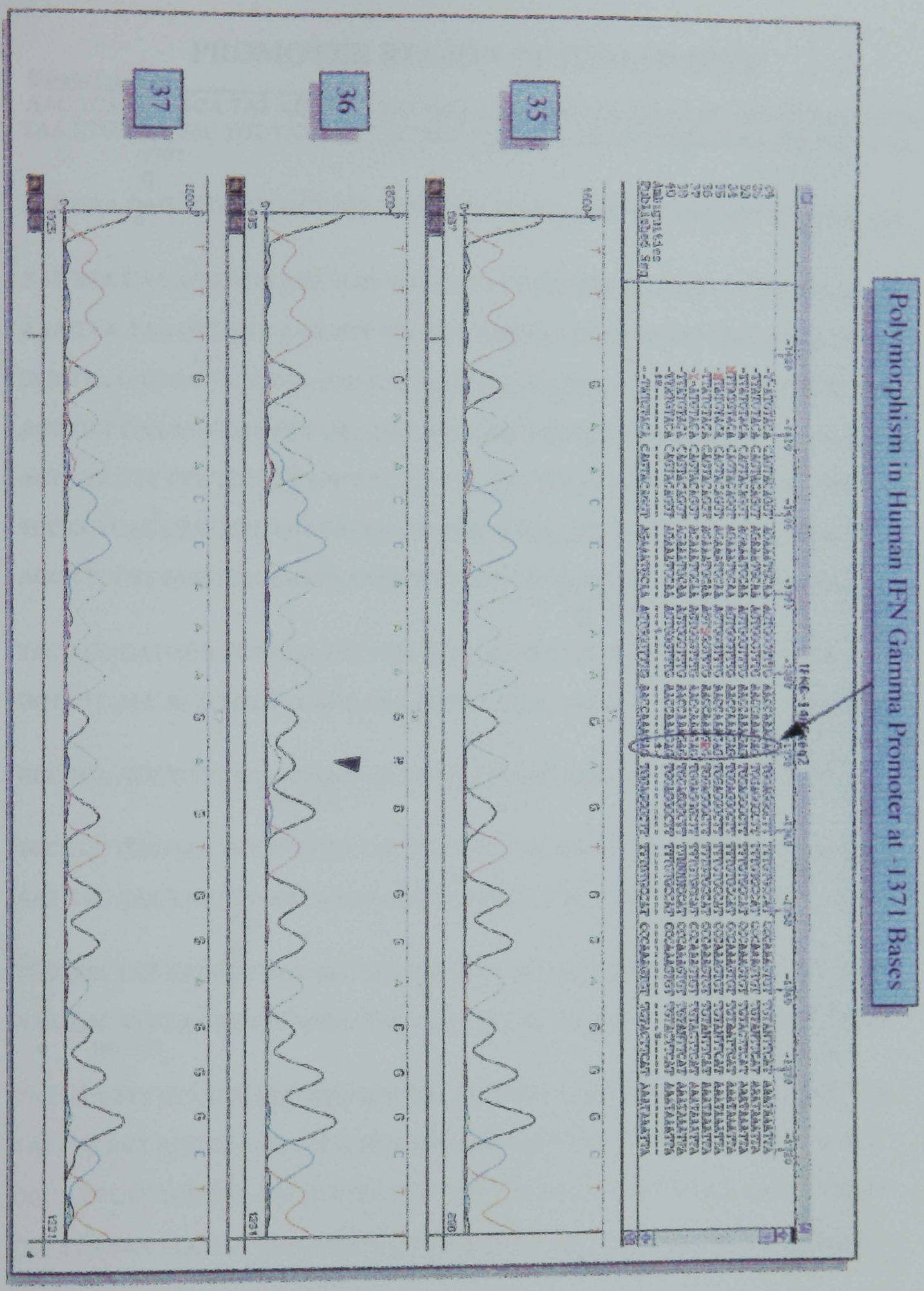


FIG. 4.6 Polymorphism of human *IFNG* promoter at -1371 bases

PROMOTER REGION OF HUMAN *IFNG*

Forward primer →

AAC TCA CAA TCA TAT AGC TAG TAT GTA GAG GAG TCA GGA ATC AAG TTT GCC CCA
TAA CTG CAA TAC TGT TAT GTA CAC AGT ACA GGT AGA AAT GCA AAG TGG GTT TGA

-1371
G

ACC AAA GAG TGG AGG GCT TTT TGT GCC ATC CCA AAG TGT TGT ACT TCA TAA ATA

-

AAT TAA CAA AGG AGG AGA AAG AAT CCT ATT TTT TTT TTG TAT CTG AAG ACA AAG

AAA TAA AAA GTT AAA AAG ATT CTC TGT TAG TAC TGA TTA TTT GGA ACA ATA AAT

TGT TTA GAG CAT TGC TGT TCA ATA TAG TAG TCA CCT AGC AGT ATG TGC CCA TTA

AGC GTT GAS ATA CGA CTA GAC CAA ATT GAG ATG CAC CGT AGG CTT AAA ATA TAC

ACT GTA TTT CTT TCC TTT TTT CTT TTT TTC TTT TTT TTT TTT GAG ACG GAA TCT TAC

TCC CGTCAC CTA GGC TGG AGT GTA GTG GCG CGA TCT CGG CTC ACT GCA ACC TCC

ACC TTT CTT GGG TTC AAG CCA TTC TCC TGC CTC AGC CTC CCT AGT AGC TGA GAT

ins -908
T

TAC AGG CAT ACA CCA CCA TGC CTG GCT AAT TTT TTG TAT TTT TAG TAG AGA TGG

GGT TTC ACC ATA TTG GCT AGG CTG GTC TCA AAC TCC TGA CCT TGT GAT CCA CCC

-790
T

GCC TCA ACC TCC CAA AGT GCT GGG ATT ACA GGT GTG AGC CAC TGC GTC TGG AAC

sub -764
C

TCC CCC TGG GAA TAT TTT CTA CAC TGT ATT TCA AGG ATT TAA TAT GAC AAA AAG

AAT GTC AAA TAC CTT ATT AAG AAT GTA GTA TAT TGA TGC ATA CTG AAG TAC TAT

-700
A

TTG GGA TAT ATT GGT TTA AAT ACA ATA TAT TTT AAA ATT ATA TTT ACC TTT TAA

AAA AAC TTT TAT TAA TGA GGC TAC TAG ATC ATT TAA ATT TAC CTG TGT GGC TTG

ins -561
T

TAT GTA TTT CTA CTG GGC AGT GCT GAT CTA GAG CAA TTT GAA ACT TGT GGT AGA

TAT TTT ACT AAC CAA CTC TGA TGA AGG ACT TCC TCA CCA AAT TGT TCT TTT AAC

CGC ATT CTT TCC TTG CTT TCT GGT CAT TTG CAA GAA AAA TTT TAA AAG GCT GCC

CCT TTG TAA AGG TTT GAG AGG CCC TAG AAT TTC GTT TTT CAC TTG TTC CCA ACC

-297
A

ACA AGC AAA TGA TCA ATG TGC TTT GTG AAT GAA GAG TCA ACA TTT TAC CAG GGC

ins -237 -234
T G

AGT GCC TCA AAG AAT CCC ACC AGA ATG GCA CAG GTG GGC ATA ATG GGT CTG TCT

-152
G

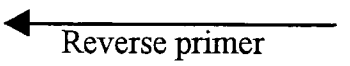
CAT CGT CAA AGG ACC CAA GGA GTC TAA AGG AAA CTC TAA CTA CAA CAC CCA AAT

GCC ACA AAA CCT TAG TTA TTA ATA CAA ACT ATC ATC CCT GCC TATCTG TCA CCA

TCT CAT CTT AAA AAA CTT GTG AAA ATA CGT AAT CCT CAG GAG ACT TCA ATT AGG

TAT AAA TAC CAG CAG CCA GAG GAG GTG CAG CAC ATT GTT CTG ATC ATC TGA AGA

TCA GCT ATT AGA AGA GAA AGA TCA GTT AAG TCC TTT GGA CCT GAT CAG CTT GAT



ACA AGA

FIG.4.7. Sequence of human *IFNG* promoter from –1487 to +66bps of initiation codon

Promoter region of interferon gamma showing all the novel polymorphisms. Polymorphisms are highlighted in red.

Key
Ins = Insertion, sub = Substitution, insertions and substitutions were observed in all samples sequenced and found to be different from already published data

4.3.2. Sequence variation in the IFN-γR1 promoter region

Five new variants of the *IFNGR1* were identified: one single point polymorphism, one deletion polymorphism and three simple point mutations.

Table 4.4. *IFNGR1* promoter variants

Substitution	Position	Genotype frequency	Allele frequency
T→C transition	-56	TT (0.23) CC (0.23) TC (0.54)	T (0.5) C (0.5)
T→G transversion	-181	TT (0.96) GG (0) TG (0.04)	T (0.98) G (0.02)
T→C transition	-270	CC (0.92) TT (0) C/T (0.08)	T (0.96) C (0.04)
Double deletion TT	-471/470	TT (0.68) -/T (0.23) -/- (0.09)	T (0.8) --(0.2)
C→T transition	-499	CC (0.97) TT (0) CT (0.03)	C (0.98) T (0.02)

Evidence for these polymorphism are documented in FIG4.9 through FIG4.13

Table 4.4 shows the type of base changes followed by the nucleotide position and then the genotype and allele frequency in parentheses.

There was an ambiguity at –38 bases of the initiation codon.. The software showed that three individuals were homozygous for adenine (A) at this position whereas twenty-five other people were shown to be homozygous for the guanine (G) at this position.

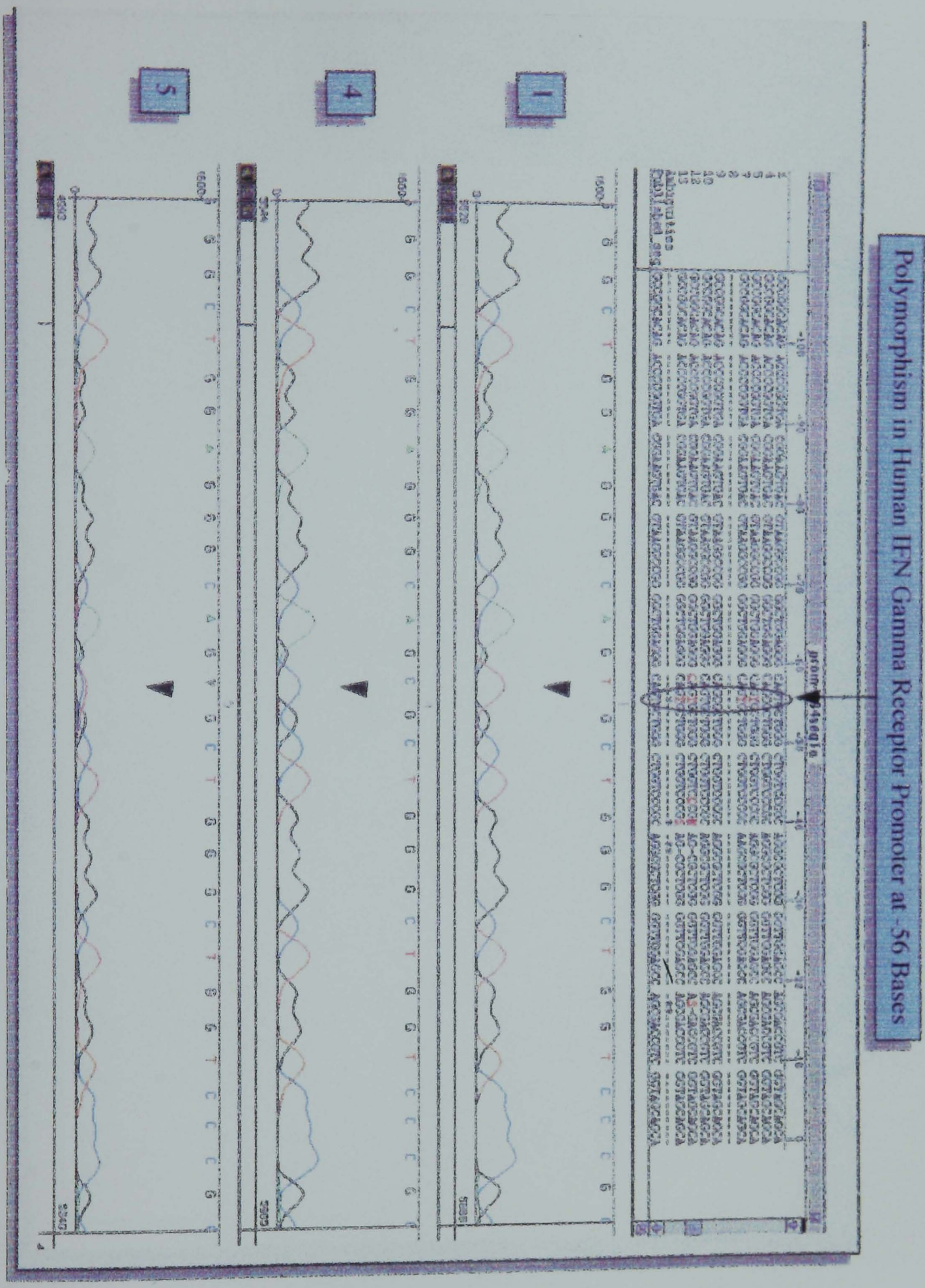


FIG. 4.8 Polymorphism of human *IFNGR1* promoter at -56 bases

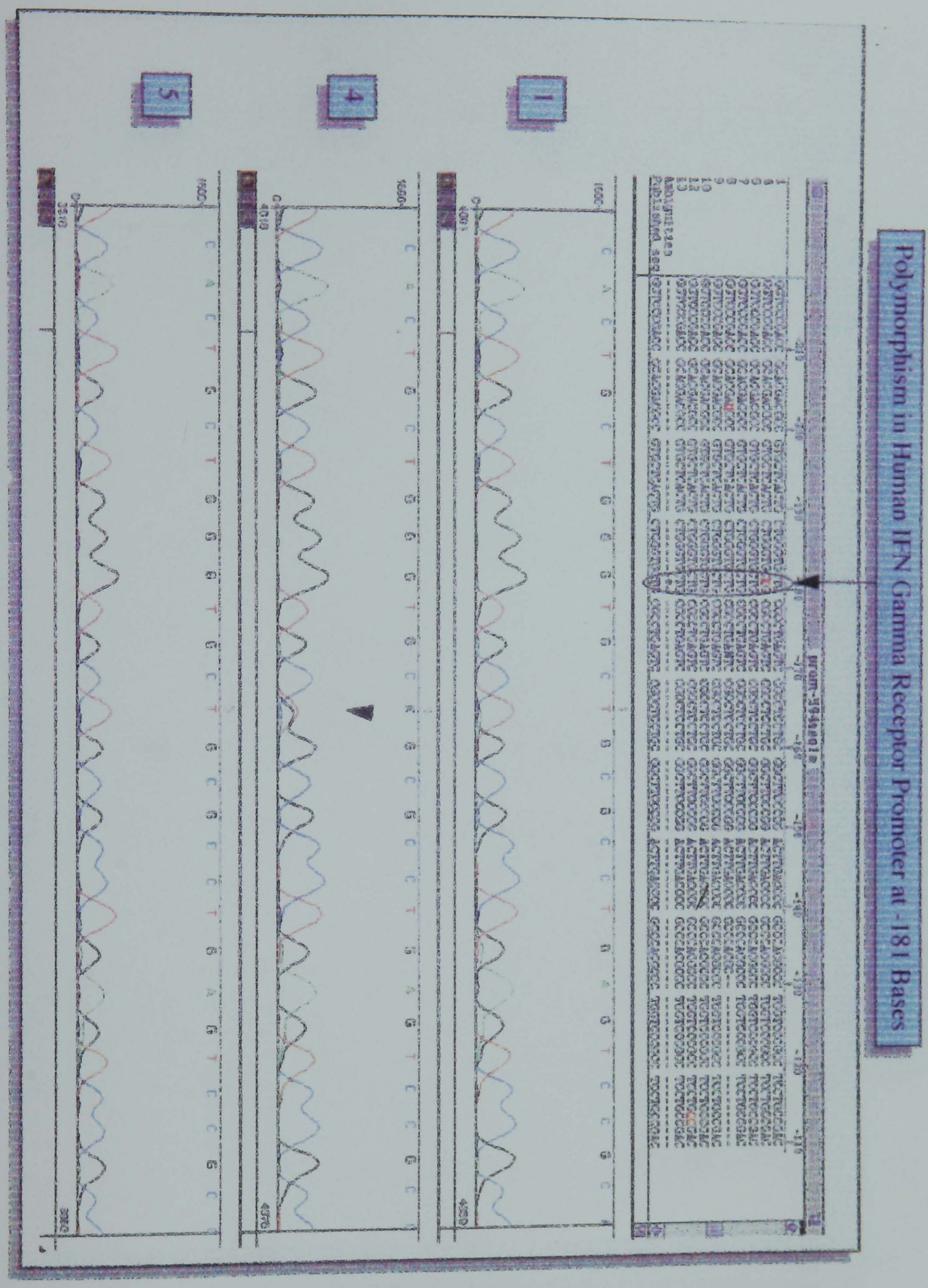


FIG. 4.9 Polymorphism of human *IFNGR1* promoter at -181 bases

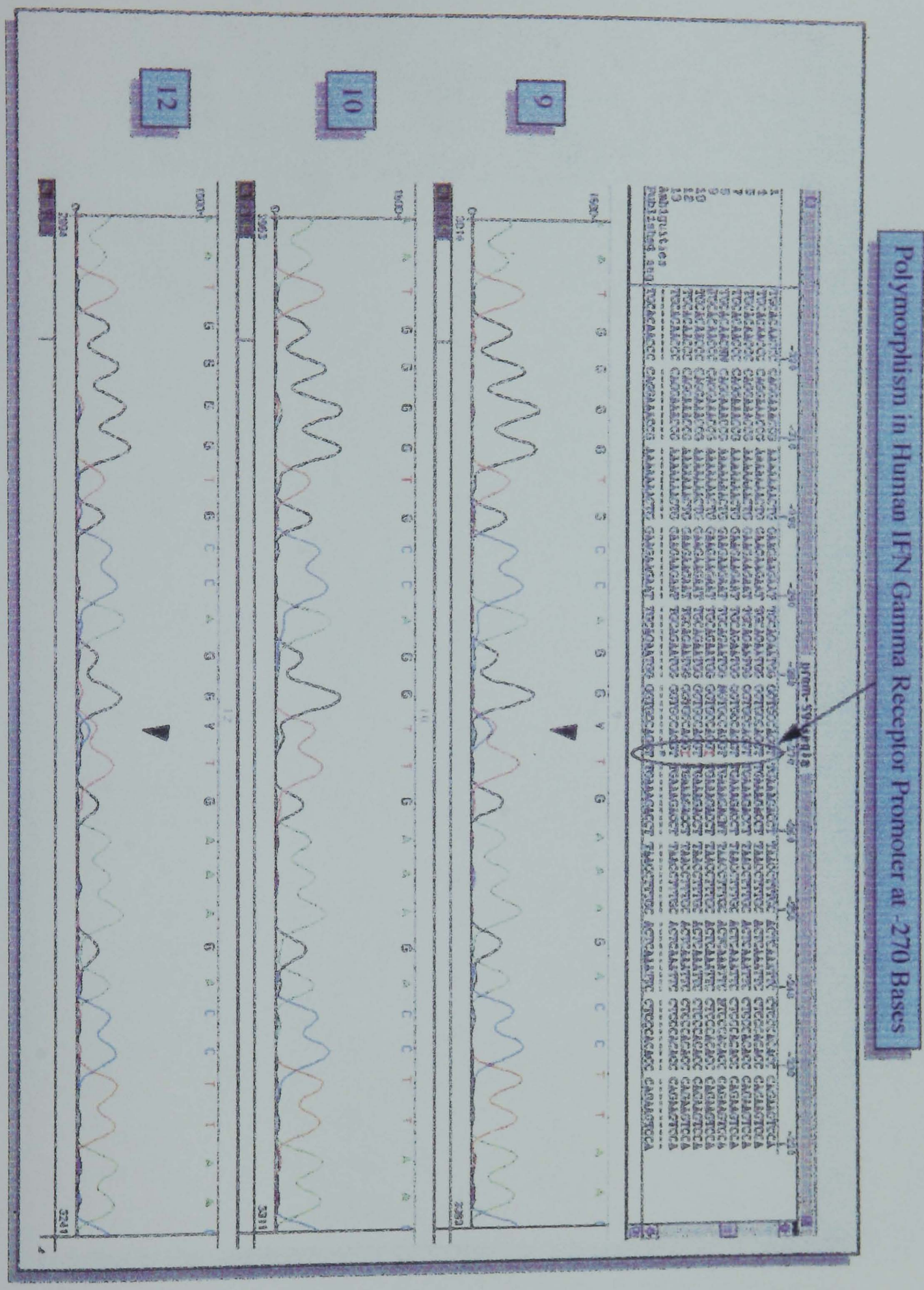


FIG. 4.10 Polymorphism of human *IFNGR1* promoter at -270 bases

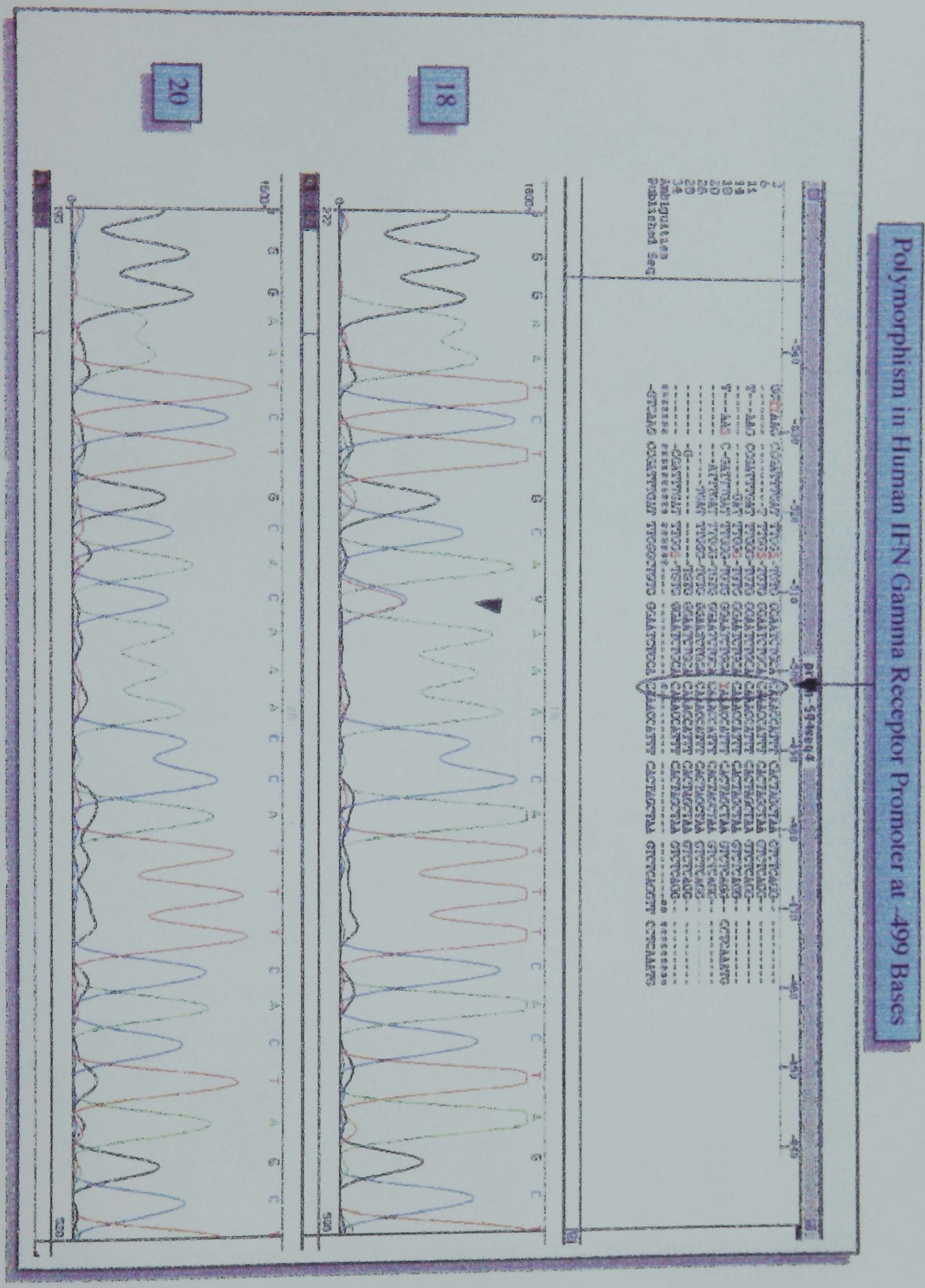
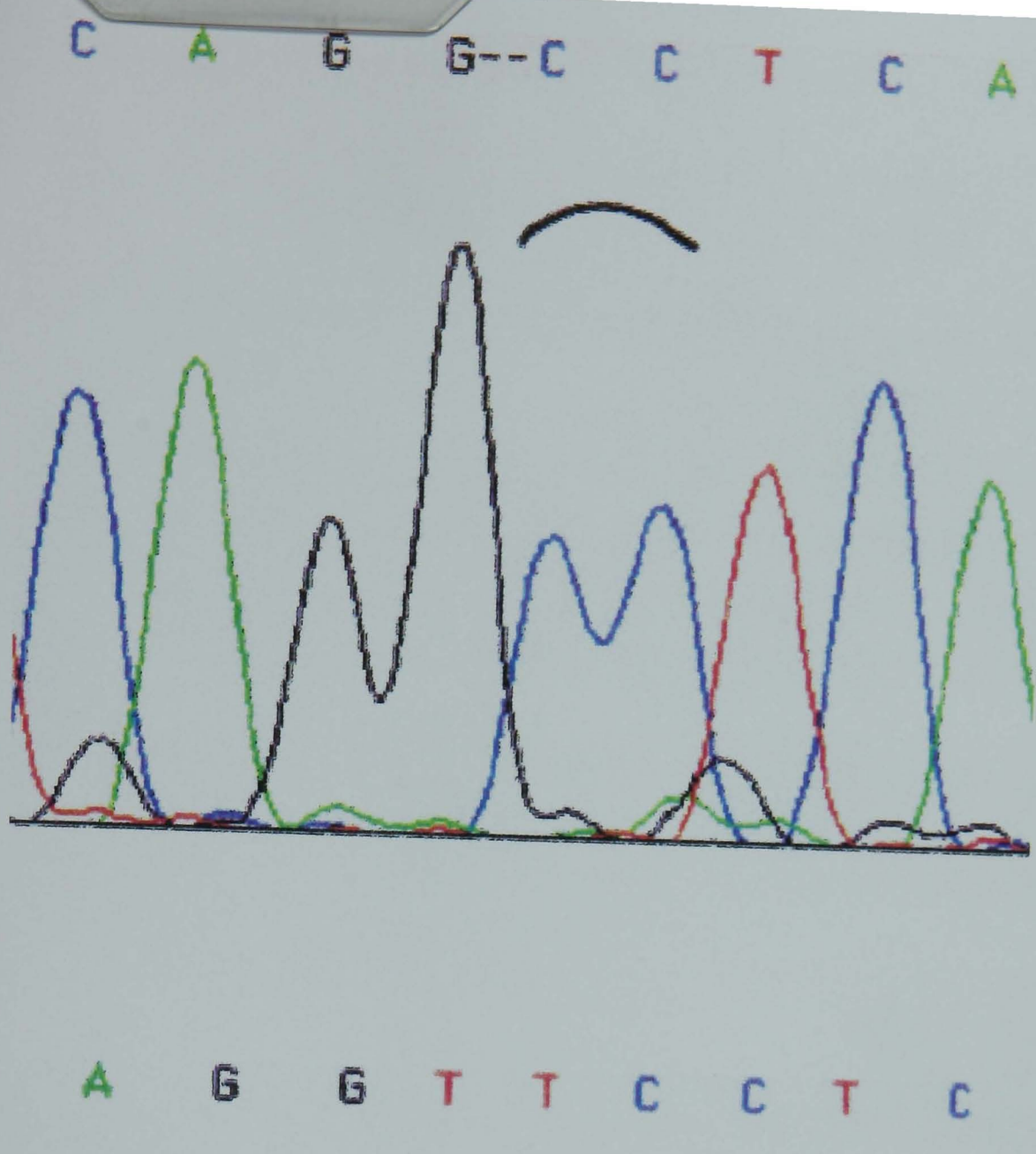
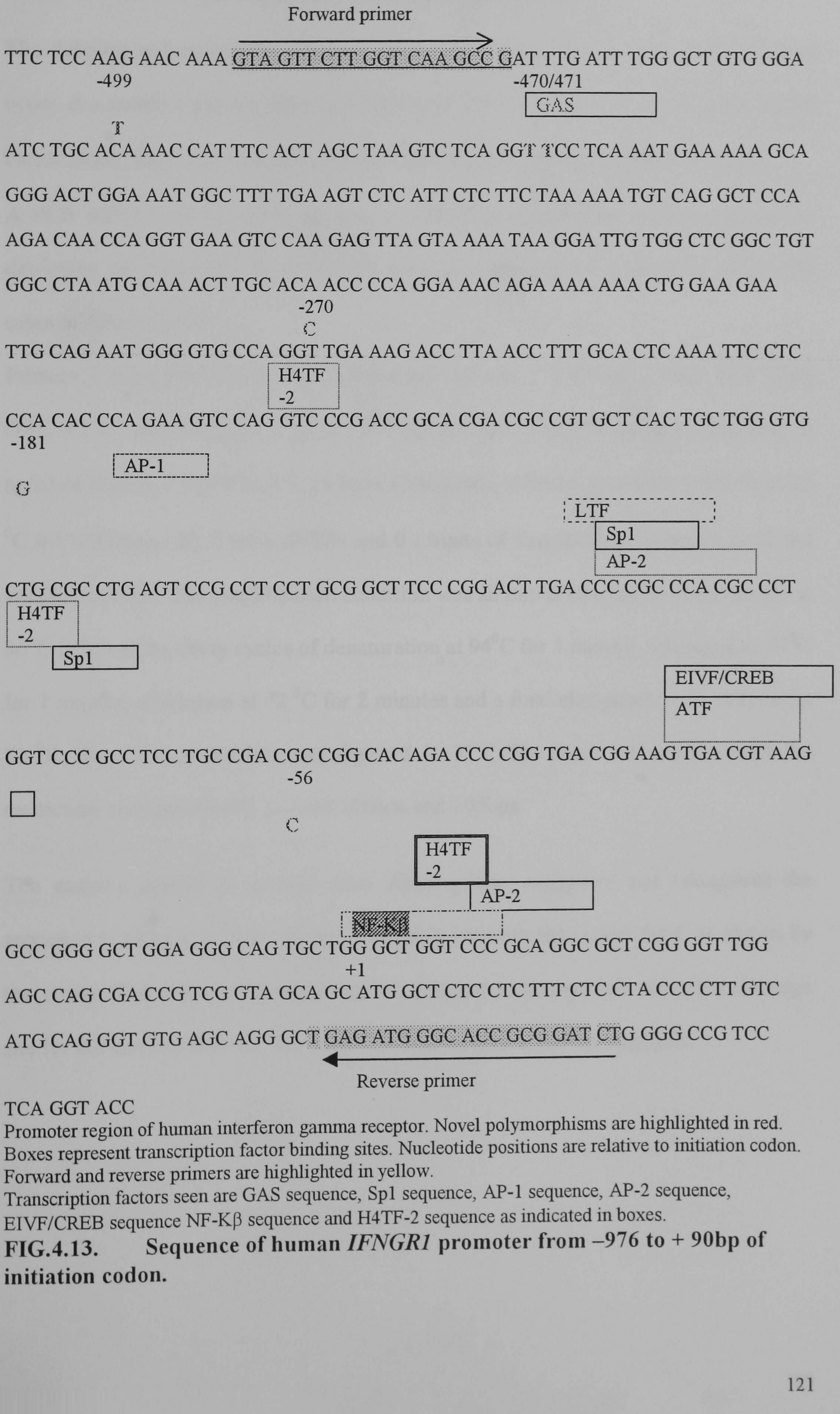


FIG. 4.11 Polymorphism of human *IFNGR1* promoter at -499 bases



G. 4.12 Double deletion in human *IFNGR1* promoter at -470/471 bases

PROMOTER REGION OF HUMAN *IFNGR1*



Promoter region of human interferon gamma receptor. Novel polymorphisms are highlighted in red. Boxes represent transcription factor binding sites. Nucleotide positions are relative to initiation codon. Forward and reverse primers are highlighted in yellow.
Transcription factors seen are GAS sequence, Sp1 sequence, AP-1 sequence, AP-2 sequence, EIVF/CREB sequence NF-Kβ sequence and H4TF-2 sequence as indicated in boxes.

FIG.4.13. Sequence of human *IFNGR1* promoter from –976 to + 90bp of initiation codon.

4.3.3. PCR RFLP OF *IFNGR1* ddel/TT polymorphism

The deletion polymorphism detected during the course of this study was found to occur at a putative gamma activation site GAS (FIG. 4.8 & 4.12). Gamma activation factor should bind to the gamma-activated site in the promoter of IFN-γ receptor.

A PCR RFLP was designed for use in screening independent control samples to determine the frequency of this polymorphism in the general population and in TB cases in The Gambia.

Primers 5'AGA GAT GCA AAA TAA CAG AC and 5' TTT ACT AAC TCT TGG GAC TT 3' were designed to give a 311 bps product. The PCR premix contained 50 ng/μl of primer, 1.5mM $MgCl_2$ 160mM $(NH_4)_2SO_4$, 670mM Tris-HCl_2 (pH8.8) at 25 ^{0}C 0.1% Tween –20, 25mM dNTPs and 0.15units of Taq DNA polymerase in 12.5μl volume reaction. The amplification condition was an initial 10 minutes denaturation at 96^{0}C followed by thirty cycles of denaturation at 94^{0}C for 1 minute, annealing at 57^{0}C for 1 minute, elongation at 72 ^{0}C for 2 minutes and a final elongation for 7 minutes at 72 ^{0}C. The 311bps product was digested at 37°C for 1hour 30 minutes with the restriction enzymes *HaeIII* to yield 206bps and 105bps.

The enzyme *HaeIII* is isolated from *Haemophilus aegyptius* and recognises the sequence 5' GG↓CC 3' The enzyme cleaves in between the G and the C as shown by the arrow. The sequence surrounding the deletion polymorphism creates a cleavage site for the enzyme *Hae III*. The deletion is cut to give a 105 bp fragment.

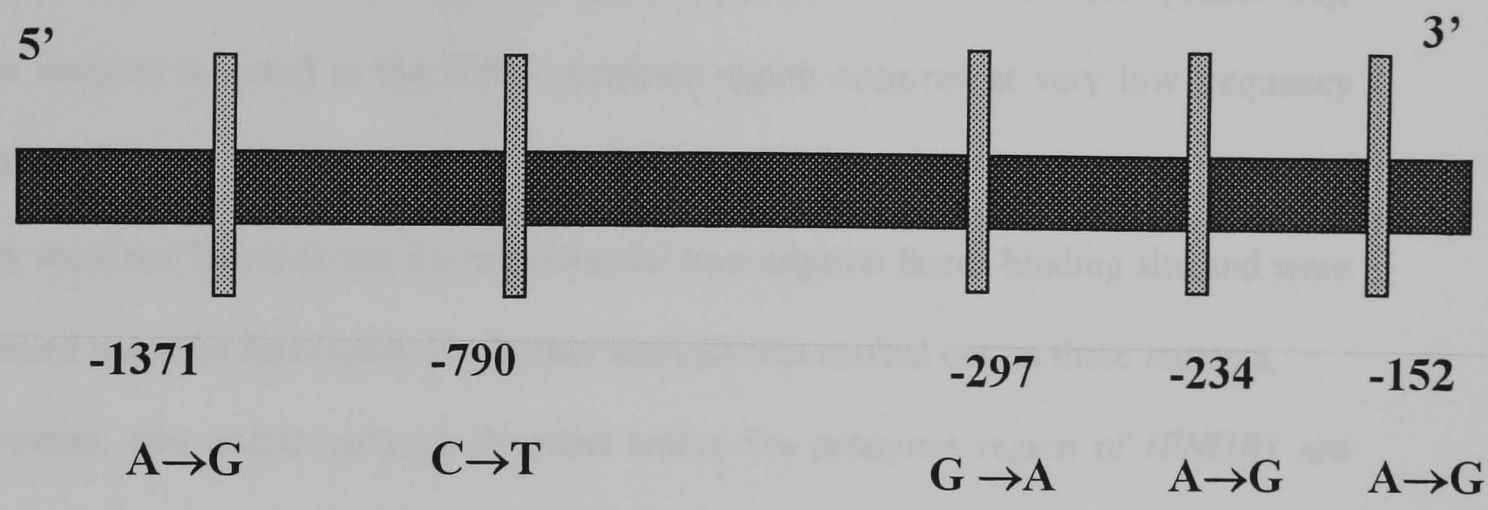
4.3.4. PCR RFLP OF *IFNGR1* –56 C/T polymorphism

A polymorphism consisting of a substitution of cytosine for thymine was observed at position –56 upstream of the transcription start site. This polymorphism is four bases away from a putative NF-Kβ binding site. An assay was design by PCR RFLP for use in screening the larger population to determine the frequency of this polymorphism in the general population and in TB cases in The Gambia.

Primers 5'GCT CAC TGC TGG GTG CTG CGC 3' and 5' AGA TCC GCG GTG CCC ATC TCA 3' were designed to amplify a product of 268 bp in size. The PCR premix contained 50 ng/μl of primer, 1.5mM $MgCl_2$ 160mM $(NH_4)_2SO_4$, 670mM Tris-HCl_2 (ph8.8) at 25 ^{0}C 0.1% Tween –20, 25mM dNTPs and 0.15units of Taq DNA polymerase in 12.5 μl volume reaction. The amplification condition was an initial 10 minutes denaturation at 96 ^{0}C followed by thirty cycles of denaturation at 94 ^{0}C for 1 minute, annealing at 57 ^{0}C for 1 minute elongation at 72 ^{0}C for 2 minutes and a final elongation for 7 minutes at 72 ^{0}C. The 268 bps product was digested at 37 ^{o}C for 1hour 30 minutes with restriction enzymes *Eco47 III* to yield 141 bp and 127 bp.

Similarly enzyme *Eco 47III* is isolated from *Eshericia coli* RFL 47 and recognises the sequence 5' AG↓C GCT 3'. The enzyme cleaves in between the C and the G as shown by the arrow. The sequence surrounding the transition polymorphism creates a cleavage site for the enzyme *Eco 47 III*. The C polymorphism is cut to give a 127 bp fragment.

a)



b)

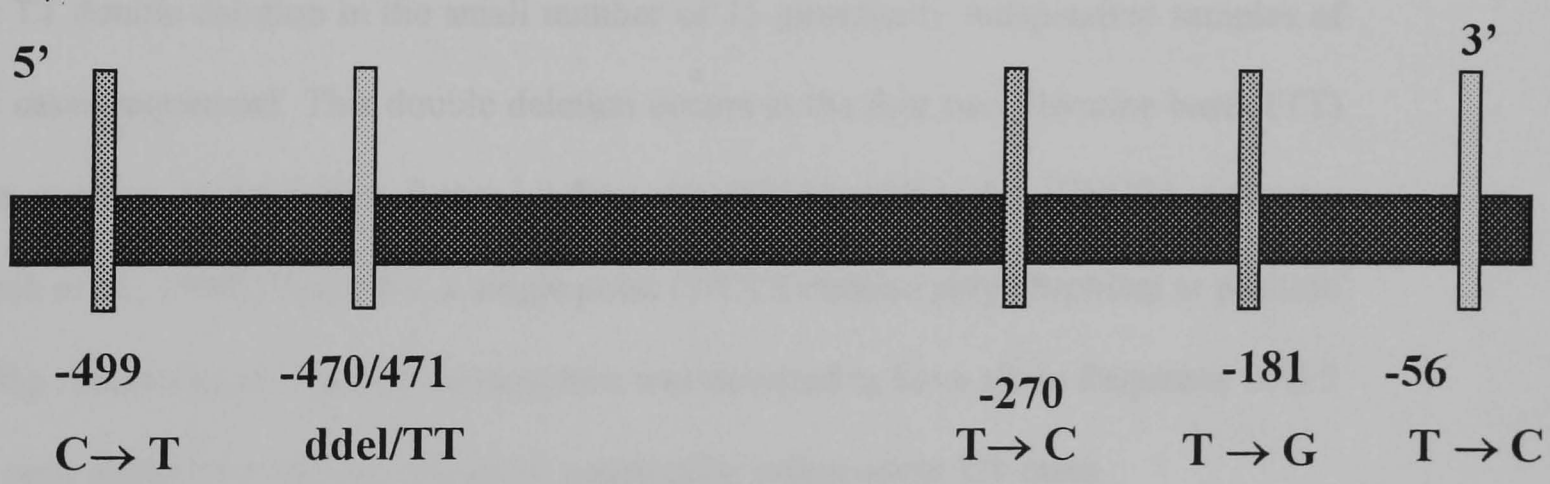


FIG. 4.14. Nucleotides changes in human a) *IFNG* and b) *IFNGR1* promoter

4.4. Discussion

Specific segments of the promoter regions of two genes *IFNG* and *IFNGR1* were sequenced in TB cases. Ten novel variants were detected. Five variants were observed within the promoter of *IFNG* (Table 4.3) and another five within *IFNGR1* (Table 4.4). The variants detected in the *IFNG* promoter region occurred at very low frequency (Table 4.3) and they may not be very useful tools for further studies. Furthermore, they were not found at any known potential transcription factor-binding site and were deemed not to be functional. No further analysis was carried out on these mutants.

However, two polymorphisms detected within the promoter region of *IFNGR1* are informative and suggest specific use in genetic studies (Figures 4.8 and figure 4.12). These are firstly, the –470/471 nucleotides double deletion polymorphism designated delTT-470/471 which has a frequency of 0.8 for the wild type allele (TT) and 0.2 for the TT double deletion in the small number of 36 genetically independent samples of TB cases sequenced. This double deletion occurs at the first two Thymine bases (TT) at a putative transcription factor-binding site (GAS) within the *IFNGR1* promoter (Bach *et al.*, 1997). Secondly, a single point (T/C) transition polymorphism at position –56bp relative to the start of transcription was detected to have allele frequency of 0.5 for each allele from sequencing of 36 genetically independent TB cases.

The promoter region of the *IFNGR*1 contains the specific consensus GAS sequence of TTNCNNNAA from -470 bases to –462 bases of the transcription start site (Bach *et al.*, 1997; figure 4.13). Sequences corresponding to this consensus GAS sequence has been used in several mobility shift assays as a ^{32} P – end labelled double stranded DNA probe (Altare *et al.*, 1998b, Jouanguy *et al.*, 2000). The double deletion of thymine (TT) at position –470/471 reported in this paper is the first two nucleotides of the GAS sequence required for early IFN-γ inducible genes at which a transcription

factor GAF binds to initiate transcription of the gene. The presence of a deletion at this site is expected to abrogate or abolish the binding of the phosphorylated homodimer STAT1 molecule which should translocate from the cytoplasm into the nucleus and bind to the gamma activated site of any γ- inducible gene. This implies that IFN-γ is capable of regulating the transcription of its own receptor α chain. A deletion at this site should therefore reduce the rate of transcription of the gene. However, there are indications from a study carried out in mice cell lines that IFNγR1 mRNA is constitutively expressed in two cell lines that utilise GAS element differently in response to IFN-γ (Lucas *et al.*, 1998).

Free NF-Kβ can translocate to the nucleus of many genes containing a kβ consensus motif (GGGRNNYYCC). The promoter region of *IFNGR1* contains a sequence (GGGCTGGTCC) at nucleotide positions –52 to – 43 of the initiation codon. This sequence differs from the consensus IKβ motif at only two points. The fourth nucleotide of this sequence is a C instead of an A or G and the seventh nucleotide is a G instead of a C or T of the consensus sequence. However, these positions could adversely affect binding of NF-Kβ and subsequent gene transcription rate (Suzuki *et al.*, 1999). Studies show that a substitution of A to G at position 4 does not contributes to affinity change because these substitutions occur in high, intermediate or low affinity NF-Kβ motifs. However, a substitution of T with G at position 7 has been shown to significantly contribute to the binding affinity of NF-Kβ. All high affinity NF-Kβ motifs have T at this position and G is associated with low affinity NF-Kβ motifs (Suzuki *et al.*, 1999). A single point (T→C) transition polymorphism at position –56bp relative to the start of transcription was detected to have allele frequency of 0.5 for each of the nucleotide from sequencing of the same 36 TB cases described above. This polymorphism is four bases upstream of the putative NF-Kβ

binding site in the promoter of *IFNGR1* gene (Figures 4.8, 4.13). Whether this polymorphism affects binding of the NF-Kβ motif is not known. PCR RFLP assay has been designed and used to determine the frequency of these polymorphisms in the larger population (section 4.3.3 and 4.3.4).

Further, biological importance of these polymorphisms maybe assessed by screening other African populations and diseases other than TB. Also, studies to carry out functional transfection assay using reporter gene construct to show whether alleles of these polymorphisms differ in their ability to drive gene expression by affecting the rate of gene transcription may be carried out.

CHAPTER: 5

INFLUENCE OF MACROPHAGE CANDIDATE GENES ON MONOCYTE FUNCTION

5.1 Introduction

Phenotypic analysis of cellular immune responses to mycobacteria has suggested that the ability to resist disease following infection depend partly on the genetic make up of the host. This is reflected in epidemiological evidence that host genetics influence susceptibility to mycobacterial diseases such as TB and leprosy, and the identification of several genes that are linked to or associated with these diseases (Newport, 1999). Host responses to mycobacteria involve complex interactions between the innate and acquired arms of the immune response (Ellner, 1997), which are described in more detail in chapter one.

M. tuberculosis is an intracellular pathogen that multiplies predominantly within the macrophage. Restriction of mycobacterial growth within this cell is therefore a crucial determinant of immunity and is essential for effective containment of the organism. Upon infection, macrophages undergo several changes collectively referred to as activation. These include increased expression of surface molecules MHC class II and cytokine receptors, up regulation of expression of NRAMP and various cytokines, and the production of ROI and RNI molecules (see section 1.2.1i). Convincing evidence that activated macrophages form a critical part in the control of *M. tuberculosis* has been clearly established by animal and in-vitro studies (e.g. Lurie *et al.,* 1952, Rook *et al.,* 1986).

Mycobacterial cell-wall components such as lipoglycans and LAM can stimulate macrophage activation by binding to the macrophage mannose receptor (Ernst, 1998), the CD14 molecule (Stuber, 1999), or the TLRs (Means *et al.,* 1999). Cytokines (particularly TNF), which mediate the initial innate immune responses to *M. tuberculosis* infection,

are important determinants in the development of *M. tuberculosis* disease and pathology (Orme & Cooper, 1999; Rook *et al.,* 1987). It is not clear why in some individuals the initial immune response controls infection while in others active disease develops. However, it is recognised that most immune responses within populations vary considerably between individuals and it is possible that the magnitude of the immune response is important. Genetic factors in turn are likely to contribute to this variation (Westendorp *et al.,* 1997).

The data presented in chapter 3 confirms that monocyte responses to LPS and LAM within a population of healthy Gambian blood donors vary considerably. The aim of the work described in this chapter was to determine the extent to which the observed variation can be attributed to host variation in candidate macrophage response genes. Candidate genes were selected on the basis of their known function in macrophage/monocyte immune responses and published evidence from other groups that genetic variation in these genes was associated with variation in immune responses. Table 5.1 summarises this rationale.

Table 5.1 Summary of selected macrophage candidate genes on the basis of function

Further details are given in section 5.1.1-5.1.8 below.

Candidate gene product	Chromosome region	Relevance of protein function	References
IL-10	1q31-q32	IL 10 is produced by macrophages and down regulates function. It is associated with reduced antimycobacterial activity and suppression of immune responses in TB	Murray *et al.*, 1997 Bousssiotis *et al.*, 2000
NRAMP1	2q35	NRAMP1 has multiple pleiotropic effects on macrophage function. It was the first murine mycobacteria susceptibility gene to be identified and variation in the human homologue has been associated with TB, leprosy and response to lepromin	Blackwell *et al.*, 1996 Bellamy *et al.*, 1998a Abel *et al.*, 1998 Alcais *et al.*, 2000
IL-1β IL-1RA	2q14	IL-1 is a pro-inflammatory cytokine, produced by activated macrophages, induces Il-2 production	Dinarello, 1996
Th2 cluster IL-4 IL-9	5q23-q32	IL-4 down regulates macrophage function. IL-4 and IL-9 promote the development of Th2 responses associated with the development of TB disease	Nicolaides *et al.*, 1997
TNF LT	6p21.3	TNF is required for protection against mycobacterial disease, but also contributes to pathology. Promoter region polymorphisms associated with functional variation in gene expression.	Knight et al. 1999 Knight and Kwiatkowski, 1999
IFNγR1	6q23-24	First human gene shown to cause increased susceptibility to mycobacterial infection Required for IFN-γ function	Newport *et al.*, 1996 Jouanguy *et al.*, 1996
IFN-γ	12q24.1	IFNγ is a potent macrophage activator.	Farrar, 1993
iNOS small inducible chemokines	17q11.2-q12	Repeated exposure of bacterial cell wall component LPS alters the composition of NF-Kβ binding to the regulatory regions of inducible nitric oxide synthase gene. There is reduced macrophage production of nitric oxide via the iNOS pathway. NO is important in killing of mycobacteria in murine macrophages. The small inducible chemokines genes are located in the same region.	Golgring *et al.*, 1998 Nicholson *et al.*, 1996

5.1.1 IL-10

IL-10 was originally described as a T-cell factor responsible for inhibition of Th1 type responses (Fiorentino *et al.,* 1989). It was subsequently shown that monocytes are also a major source of IL-10, that IL-10 production is down regulated by IFN-γ and that IL-10 down regulates monocyte function (de Waal Malefyt *et al.,* 1991). In 1997, Turner *et al.,* demonstrated a difference in serum IL-10 in association with the presence or absence of A at position –1082 of the human *IL-10* promoter following ConA stimulation of peripheral blood mononuclear cells. Also, allele 7 of the proximal and 3 of the distal microsatellites identified within the *IL-10* gene promoter region are associated with decreased expression of IL-10 protein whereas alleles 14 of the proximal and 2 of distal repeat were associated with increased IL-10 expression (Eskdale *et al.,* 1998).

5.1.2 NRAMP1

In mice, Nramp1 regulates a cascade of gene-inductive events mediating inflammation, elimination of invading organisms and induction of T cell memory against re invasion (Blackwell *et al.,* 1996). Nramp1 appears to influence the polarization of Th cell development in mice (Soo *et al.,* 1999), and development of delayed type hypersensitivity reactions to lepromin in humans (Alcais *et al.,* 2000). Reporter gene construct studies suggest a correlation between variations in a functional repeat polymorphism in the promoter region of human *NRAMP1* infectious versus autoimmune disease susceptibility (Searle & Blackwell, 1999). Various genetic studies have identified either linkage or association with mycobacterial disease in humans and *NRAMP1* (Bellamy *et al.,* 1998, Abel *et al.,* 1998, Greenwood *et al.,* 2000).

5.1.3 The IL-1β and IL-1RA gene cluster.

IL-1β is a potent proinflammatory cytokine implicated in numerous physiological processes, and in the pathogenesis of a number of inflammatory disorders (Dinarello, 1996). IFN-γ increases IL-1β production while IL-10 down-regulates IL-1β production. Allele 1 (T) at position +3953 in *IL1B* has been associated with increased expression of IL-1β (Pociot *et al.,* 1992). IL-1 receptor antagonist (IL-1RA) is one of the most powerful endogenous anti-inflammatory agents known and competes with IL-1β and IL-1α for occupancy of its cell surface receptors. Since it does not stimulate signal transduction, it acts as an inhibitor of IL-1 action (Blakemore *et al.,* 1995). Intron 2 of *IL1RN* contains an 86bp VNTR repeat of which the allele 2 is associated with increased expression of IL-1RA by monocytes in vitro (Tarlow *et al.,* 1993).

5.1.4 Th2 cytokine cluster

Several studies have revealed tight linkage between markers on human chromosome 5q31.1 and a major susceptibility gene controlling total serum IgE levels. Naive CD4 T cells develop into one of two major subsets of Ths that produce distinct sets of cytokines Th1 (IFNγ, IL-12 and TNF-α) and Th2 (IL-4 IL-5, IL9, IL10) and IL-13 cell subsets. The polarisation of responses to Th1 or Th2 dominance often determines resistance or susceptibility of host to infection and the degree of tissue damage in many autoimmune diseases. The region of Human chromosome 5q31 encodes the cluster of Th2 cytokine genes. These cytokines include IL-4, IL-5, IL-9 and IL-13. Th2 cytokines are associated with antibody production and high serum IgE levels. The hypothesis that: A raise in atopic disorders in affluent communities correlates with a decline in infectious disease, is

supported by several studies (Shirikawa *et al.,* 1997, Shaheen *et al.,* 1996, Seah *et al.,* 2000, Aung *et al.,* 2000). Some of these studies show that Th2 cytokines are associated with immunopathologic changes in TB patients and could be a cause or consequence of disease. Transgenic mice models where IL-4 and IL-9 are either over expressed or where the gene is disrupted by recombination have been used to show the importance of IL-4 in both IgE synthesis and allergic disease (Song *et al.,* 1996, Nicolaides *et al.,* 1996). Cells isolated from atopic individuals have the ability to over express the IL-4 and IL-9 gene relative to nonatopic individuals (Song *et al.,* 1996). Allelic forms of the *IL4* promoter region account for the higher transcriptional activation in atopic patients (Song *et al.,* 1996). Functional studies in B6 mice showed that alterations of a quantitative trait locus that maps to mouse chromosome 13 encoding the IL-9 was associated with reduced expression of IL-9 and bronchial hypo responsiveness (Nicolaides *et al.,* 1997). Perhaps, cells from TB patients have increased capacity to produce both IL-9 and IL-4. Significant inhibition of allergen-specific IgE production, OVA-induced eosinophilia and goblet cell development by prior infection with BCG was observed by Yang *et al.,* (1999). In addition, intracellular bacterial infection (i.e. BCG) can inhibit antigen-specific IgE and airway reactivity induced environmental allergen (Yang *et al.,* 1999). The effect of two polymorphic variants of *IL4* and one of *IL9* on cytokine levels was investigated in Gambians.

5.1.5 TNF and lymphotoxin-α (LTα)

TNF plays a critical role in lymphocyte biology as well as in a variety of infectious and autoimmune diseases (Beutler, 1989). It is produced by a variety of cell types and is one

of the first immunological mediators to appear in response to infection. Resting macrophages produce virtually no TNF: however, monocytes and macrophages produce large amounts of this cytokine within hours of stimulation with an appropriate agent and IFN-γ potentates its release (Renz *et al.,* 1988). Stable inter-individual variations in plasma TNF levels have been observed suggesting heritable differences in TNF production (Molvig *et al.,* 1988, De Groote *et al.,* 1992). A number of polymorphisms have been identified in the promoter region, and because production of TNF protein is regulated in part at the transcriptional level, there has been much interest in the effect of these polymorphisms on this process. The *TNFA* –308 polymorphism is perhaps the best studied. However, there are contradictory reports of the same allele being associated with both high and low TNF production, presumably due to the experimental protocol followed (reviewed by Knight and Kwiatkowski, 1999). The most convincing data relates to a single nucleotide polymorphism at position *TNFA*–376 relative to the transcriptional start site. The mutant allele completes a transcription factor OCT-1 binding site. This site is a region of complex protein–DNA interaction such that binding alters gene expression in human monocytes resulting in four fold increased susceptibility to cerebral malaria (Knight *et al.,* 1999).

An increase in LT and TNF secretion is associated with a SNP located in intron 2 of the *LTA* (Messer *et al.,* 1991, Abraham *et al.,* 1993). Stuber *et al.,* (1996, 1999) found the same allele to be associated with high circulating concentrations of TNF and higher mortality for septic patients, despite showing no effect for the *TNFA*-308 alleles on circulating TNF levels.

5.1.6 IFN-γ

IFN-γ is the major cytokine required for macrophage/monocyte activation, acting via its heterodimeric receptor (Farrar, 1993) and a large body of data suggests it is essential for mycobacterial immunity in both mouse and man. Intron one of the IFN-γ gene contains a variable CA repeat element (Ruiz-Linares *et al.,* 1993). In vitro production of IFN-γ showed significant correlation with the presence of allele 2 (Awad *et al.,* 1999, Pravicia *et al.,* 1999) suggesting IFN-γ production is genetically regulated. Novel promoter polymorphisms have been identified in this gene in the Gambian population studied (see chapter 4).

5.1.7 IFNγR1

Inherited IFN-γ R1 deficiency has been identified as the cause of disseminated mycobacterial infection in several kindred (Newport *et al.,* 1996, Jouanguy *et al.,* 1996), highlighting the importance of this pathway in mycobacterial immunity. Novel polymorphisms in the *IFNGR1* gene were identified (chapter 4) and studied subsequently in relation to immune responses in the Gambian population.

5.1.8 Inducible nitric oxide synthase (iNOS)

LPS induces iNOS expression in several cell types including macrophages and is regulated by several cytokines in a species-dependent manner (Nathan *et al.,* 1994). Functional studies show that the critical control element appears to be clustered within the first 1700bp region 5' of the transcriptional start site (Xie *et al.,* 1994). Activation of human promoter–reporter occurred with either IL-1β or TNF alone but IFN-γ was

absolutely required for the production of NO (Lowenstein *et al.*, 1993). The ability of differing numbers of (CCTTT) n pentanucleotide repeats to induce transcription of *NOS2A* gene has been analysed using a reporter gene assay in transfected colonic carcinoma cells (Warpeha *et al.*, 1999). IL-1β induction was most effective in constructs carrying the 14 repeat allele. The carriers of this 14 repeat allele also had lower incidence of renal complications and diabetes (Warpeha *et al.*, 1999).

5.1.9 Small inducible chemokines

Chemokines that are preferentially chemotactic for macrophages have been implicated in the immune response in tuberculosis (Riedel *et al.*, 1997, Orme and Cooper, 1999). Individual chemokine genes are differentially regulated in response to LPS (Kopydlowski *et al.*, 1999).

5.2 Methods

In order to determine the influence of macrophage candidate genes on monocyte function, cytokine production was measured using a whole blood assay in 312 healthy blood bank donors, as described in chapters 2 and 3. The cytokines measured were pro inflammatory TNF and IL-1β, and anti-inflammatory IL-10 after stimulation with bacterial endotoxin LPS and mycobacterial LAM. Published polymorphisms at several macrophage candidate gene loci were genotyped as described in chapter 2 and below. The influence of polymorphisms at these macrophage candidate gene loci on macrophage function (cytokine release) was determined using a non-parametric, one-way analysis of variance test.

5.2.1 Whole blood assays

Whole blood assays were carried out as described in chapter 3 section 3.2.3. The cytokines measured were TNF, IL-1β and IL-10, all of which are involved in monocyte immune responses to *M. tuberculosis*. Furthermore, there is evidence that production of these cytokines is genetically regulated in other populations (Molvig, 1988, Eskdale *et al.,* 1998). Cytokine production was measured in response to LAM or LPS stimulation, with and without IFN-γ priming.

5.2.2 DNA genotyping methods

All genotyping was done using the polymerase chain reaction (PCR) as described in chapter 2 section 2.3.2. Various genotyping methods were employed during the course of this project: - microsatellites were analysed using the fluorescent-based semi-automated technology developed by Perkin Elmer, while SNPs were typed using restriction enzyme digestion or ARMS PCR.

5.2.2i Microsatellite analysis.

The PCR conditions were generally the same for all microsatellite polymorphisms analysed and have been described in chapter two. However, primer concentrations did vary for each microsatellite and are listed below. One primer of each pair was labelled with a 2'7'-dimethoxy-4', 5'-dichloro-carboxy fluorescein compound to the 5' end. Three fluorescein dyes were used FAM™ for Blue, NED™ for yellow and HEX™ for green. Typically, labeled primers were used at lower concentrations than the unlabelled complimentary primer. Each microsatellite was amplified separately and the PCR products pooled for gel electrophoresis. Electrophoresis was performed using the ABI PRISM system as described in section 2.3.2vii. The primer sequences and annealing

Marker	Primer sequences (5'-3')	Ta in C	Size (bp)	No of Alleles
IL10	FAM-GGTAGAGCAACACTCCTCGC GTTGTAAGCTTCTGTGGCTGG	60	250-300	11
NRAMP1	NED-GAGGGGTCTTGGAACTCCA TACCCCATGACCACACCC	58	119-123	3
D2S1471	FAM-TACCATTCACACATCGAAGAAC GATCTCTCCCCCACAATAAAC	50	80-110	20
IL4	HEX-AGCCATCTCGGTTGGATGGA CTCAAAGTGCTGGGATTAGC	55	175–219	21
IL9	FAM-CTAATGCAGAGATTTAGGGC GTGGTGTAAAGACTGCATAG	50	126-146	12
TNFa	HEX-TGTGTGTTGCAGGGGAGAGAGG CCTCTCTCCCCTGCAACACACA	60	101-127	13
IFNGR1	FAM-GAT TTG TGT ATT GCT TTA AGC GGC TCT TCA GCC TAT ATT TCC	50	180-210	14
IFNG	NED-GCT GTC ATA ATA ATA TTC AG GCC TTC CTG TAG GGT ATT AT	50	142–158	9
NOS2A	NED-CCCCTGGAAGCCTACAACTGCAT CCACTGCACCCTAGCCTGTCTCA	50	172-217	15
D17S250	HEX-GCTGGCCATATATATATTTAAACC GGAAGAATCAAATAGACAAT	54	138–172	18

Ta represents annealing temperature per primer, C represents degree Celsius

Table 5.2.1 Microsatellite primer sequences

temperatures for each microsatellite are shown in table 5.2.1. Figure 5.1 shows a typical gel electrophoresis performed as described in section 2.3.2vii. The use of different coloured fluorescent dyes for PCR products of similar sizes allows the 10 sets of PCR products to be analysed on one gel. The red bands represent internal size standard to allow accurate sizing of PCR products required for genotyping.

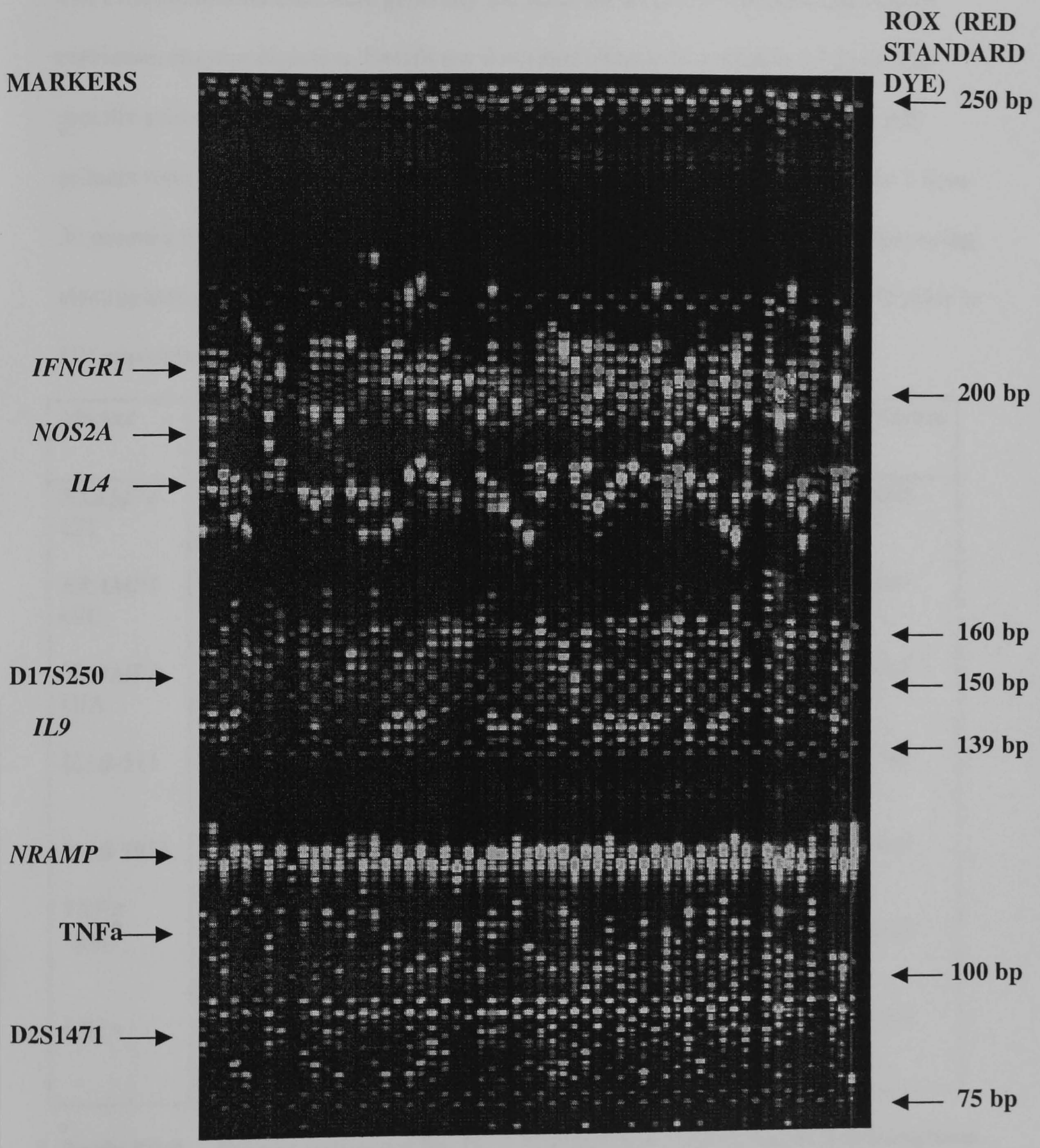


Figure 5.1 Typical ABI genescan image showing microsatellite alleles for candidate genes described in table 5.1. *IFNGR1* (blue), *NOS2A* (yellow), *IL4* (green), D17S250 (green), *NRAMP1* (yellow), TNFa (green), D2S1471 (blue). *IL9* (blue) is omitted on this gel due to technical failure. Thr standard ROX dye is red.

5.2.2ii Restriction fragment length polymorphism (RFLP) analysis

The PCR conditions used were generally the same for all polymorphisms analysed by restriction enzyme digestion. Details are described chapter two, section 2.3.2viii. The specific primers used and annealing temperatures used are shown in table 5.2.2. All primers were used at a concentration of 50 ng/ul. PCR products were digested for 1 hour 30 minutes with the appropriate restriction enzyme and the products visualised following electrophoresis thorough 3-4% agarose. DNA was stained with ethidum bromide prior to UV exposure.

Marker	Primer sequences (5'-3')	Ta (°C)	Size (bp)	Enzyme
NRAMP1 C/T	TGCCACCATCCCTATACCCA TCTCGAAAGTGTCCCACTCAG	60	216	*MnlI*
NRAMP1 G/C	TCTCTGGCTGAAGGCTCTCC TGTGCTATCAGTTGAGCCTC	64	624	*ApaI*
NRAMP1 G/A	GCAAGTTGAGGAGCCAAGAC ACCTGCATCAACTCCTCTTC	64	241	*BsrI*
IL1B-511	TGGCATTGATCTGGTTCATC GTTTAGGAATCTTCCCACTT	55	304	*AvaI*
IL1B 3953	CTCAGGTGTCCTCGAAGAAATCAAA GCTTTTTTGCTGTGAGTCCCG	55	182	*TaqI*
TNFA -308	AGGCAATAGGTTTTGAGGGCCAT TCC TCCCTGCTCCGATTCCG	60	107	*NcoI*
LTA	CCGTGCTTCGTGCTTTGGACTA AGAGCTGGTGGGGACATGTCTG	60	740	*NcoI*

Table 5.2.2 Primer sequences for PCR restriction fragment length polymorphism.

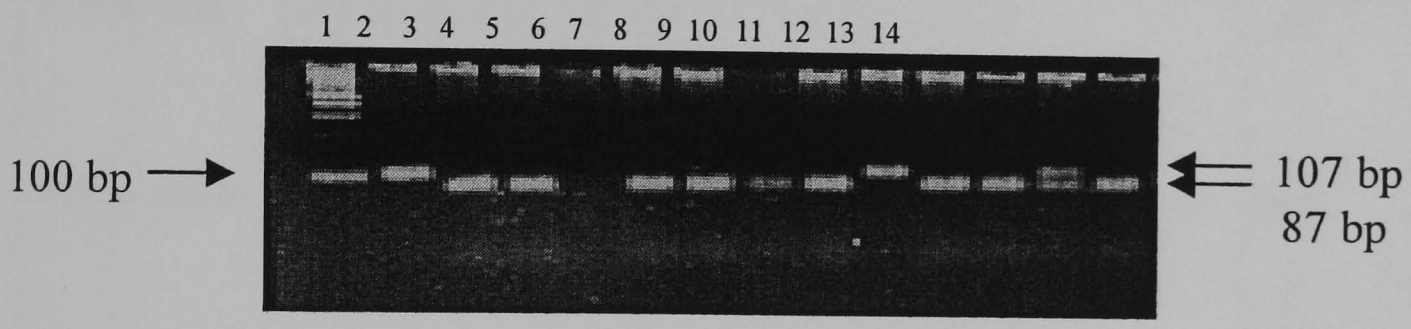


Fig. 5.2 Restriction fragment length polymorphism at position -308 in the *TNFA* promoter region

PCR products were digested with the restriction enzyme *NcoI* for 1.5 hours and then electrophoresed through a 4% agarose gel containing 0.5 mg/ml ethidium bromide. DNA was visualised with UV light and photographed. Lane 1 represents a 100 bp DNA ladder. *Nco*I cuts in the presence of the wild type allele 1 (G at position -308) and lanes 3 and 4 for example are homozygotes for the wild type allele. In contrast, lane 10 is homozygous for the mutant allele and the PCR product remains undigested. Lane 13 is heterozygous.

5.2.2iii Amplification refractory mutation system PCR

Anna Richardson in Professor Kwiatkowski's laboratory in Oxford provided primers for amplification refractory mutation system (ARMS) PCR. Primers were designed to use a conserved primer together with sequence specific primers for either of the TNF mutant and wild type alleles as listed below (Table 5.2.3) with the respective product size. The method for carrying out ARMS PCR is explained in section 2.3.2vii.

Markers	Primer sequences	Size (bp)
TNFA–238	Wild type 5'CCCCATCCTCCCTGCTCC 3', Mutant 5'CCCCATCCTCCCTGCTCT 3' Consensus 5'GGGGTCTGTGAATTCCCGG3'	395
TNFA –376	Wild type CCTGCATCCTGTCTGGAAG3', mutant 5'TCCTGCATCCTGTCTGGAAA3' consensus 5'GGCTGGGTGTGCCAACAAC3'	396
TNFA –857	Wild type 5'TCTACATGGCCCTGTCTTCG3' mutant 5'TCTACATGGCCCTGTCTTCA3' Consensus 5' AAGGATAAGGGCTCAGAGAG	270 263
TNFA–863	Wild type 5'CGAGTATGGGGACCCCCC 3', mutant 5,GAGTATGGGGACCCCCA 3' Consensus 5' CCGGGAATTCACAGACCCC3'	
HLA class 2 *DRB1*Exon 3	5' TGCCAAGTGGAGCACCCAA 3' 5' GCATCTTGCTCTGTGCAGAT 3'	796

Table 5.2.3 ARMS PCR primer sequences

The 3' mismatch primers are underlined for the mutant allele. A 796 bp fragment of the HLA *DRB1* gene was co-amplified for each PCR reaction as a positive control.

Fig. 5.3 Amplification refractory mutation systems at position - 863 in the *TNFA* promoter region.

Amplification of each DNA template was carried out using three primers. Two primers were designed such that each one contained a matching nucleotide at the 3' end corresponding to either the wild type variant of the mutant variant. Each of these primer is expected to anneal to a common complimentary DNA sequence. Each template DNA was amplified in two wells. On this gel picture there are 6 individuals. The odd numbered wells represents the wild type allele and the even numbered wells represents the mutant allele. For example, wells 1 and 2 represent DNA from the same individual and wells 3 and 4 from another individual etc.

Each well contains control primers designed to amplify exon 3 *DRB1* HLA2 and should not compete with the allele specific primers.

Samples TB 363, TB 362, TB 361 are homozygote for the wild type, while TB 360 is a heterozygote and TB 359, TB 358 are homozygote for the mutant allele.

5.2.2iv PCR VNTR

The PCR was carried out as described in chapter two and there was no further analysis because band sizes were large enough to be resolved on a 2% agarose gel. Below (Table 5.2.4) are the primer sequences used, annealing temprature (Ta) and expected band sizes.

Markers	Primer sequences	Ta in C	Size bp
IL1RN	5'CTC AGC AAC ACT CCT AT3' 5' TCC TGG TCT GCA GGT AA3'	54°	240-595
IL4 P2	5' CTG TTC ACC TCA ACT GCT CC 3' 5' TAG GCT GAA AGG GGG AAA GC 3'	65°	123-253

Table 5.2.4 PCR VNTR primer sequences

All products from PCR RFLP, VNTR and ARMS PCR were resolved in 2% agarose gel, stained with ethidium bromide, and visualised under UV light.

5.2.3 Statistical analysis

The Kruskal Wallis test of variance analysis, a non-parametric test was used to test if the cytokine measurement differed significantly between genotypes (see section 2.4). P valves of < 0.05 were taken as indicating significance. Statistical analysis was done using Stata (version 5 Stata Corporation, College Station, TX).

Table 5.3 List of macrophage candidate genes genotyped

Chromosome region	Candidate gene/marker	Polymorphism
1q31-q32	*IL10*	5' proximal promoters (CA) n repeats
2q35	*NRAMP 1*	5' promoter t (gt) 5ac(gt) 5ac(gt) $_{ng}$ or t (gt) 5ac(gt) $_{n}$ g Intron 4 PCR.RFLP Intron 13 PCR.RFLP 274 C/T Exon 3 PCR RFLP
2q14	*IL1B* *IL1RN*	Exon 5 +3953 PCR RFLP Promoter –511 PCR RFLP Intron 2 86bp VNTR
5q23-q32	*IL4* *IL9*	Intron 2 70 bp VNTR Intron 3 (GT) $_{n}$ repeat (ABI) Intron 4 (TG)n repeat (ABI) Exon 5 (T113M)
6p21.3	*LTA* *TNFA*	Intron 2 / Exon 3 PCR RFLP -863 bp G/A SNP -857 bp G/A SNP -376 bp G/A. SNP -308 bp G/A SNP -238 bp G/A SNP TNF (AC) $_{13}$ (ABI)
6q23-24	*IFNGR1*	Promoter –470del/TT Promoter –56 C/T Intron 6 (GT) $_{n}$ repeat
12q24.1	*IFNG*	Intron 1(CA) $_{n}$ repeat
17q11.2-q12	*NOS2A* D17S250	(CCTTT) $_{n}$ repeat (ABI) (AC) $_{25}$ (ABI)

5.3 Results

Twenty- five polymorphisms at eight macrophage candidate genes were investigated for influence on microbial induced responses. These genes encode proteins that are involved in macrophage activation and they will be used to determine the human genetic background responsible for inter individual differences in cytokine responses to TB. This should ultimately make clearer the understanding of the influence of genomic variation on gene regulation and cytokine protein expression on TB.

The result were presented by stratifying genotypes of a particular candidate gene and mean cytokine production in response to stimulants; LAM and LPS in the presence or absence of IFNγ. Cytokines measured are TNF, IL-1β, and IL-10. The data for chi-square, degrees of freedom and p-value is presented per cytokine and stimulant. A p-value of 0.05 or below was considered significant. No correction for multiple testing was carried for influence of genotype on quantitative trait measurements in this chapter.

χ^2: Chi-Square, D.F.: degrees of freedom and p: p value (conditional probability). LPS: Lipopolysaccahride, LAM: Lipoarabinomannan and IFNγ: interferon gamma.

5.3.1i *NRAMP1* (CA repeat, Z DNA promoter polymorphism)

Four polymorphisms in the *NRAMP1* gene were typed and analysed in relation to cytokine production. There was no quantitative trait measurement for LAM induced responses for those with the genotype 1.2. P-valves were compared between four genotypes for LPS induced by cytokine. Only two genotypes were analysed for LAM induced responses.

None of the alleles of the promoter repeat were shown to influence cytokine production.

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	1.57	2	0.5
IL-1β		0.02	2	0.99
Il-10		1.56	2	0.45
TNF	LAM&IFN-γ	2.26	2	0.3
IL-1β		0.82	2	0.66
IL-10		0.51	2	0.78
TNF	LPS	3.58	3	0.3
IL-1β		3.69	3	0.29
IL-10		3.12	3	0.37
TNF	LPS&IFNγ	1.66	3	0.65
IL-1β		3.42	3	0.33
IL-10		3.16	3	0.37

Table 5.3.1i *NRAMP1* (Z DNA polymorphism CA repeat)
Table gives statistics for influences of NRAMP1 Z DNZ polymorphism genotypes on cytokine (TNF, IL-1β and IL-10) release by stimulants.

5.3.1ii *NRAMP1* (274 C/T exon 3)

P valves were compared between three genotypes for each cytokine and stimulant.

None of the alleles of the promoter repeat were shown to influence cytokine production.

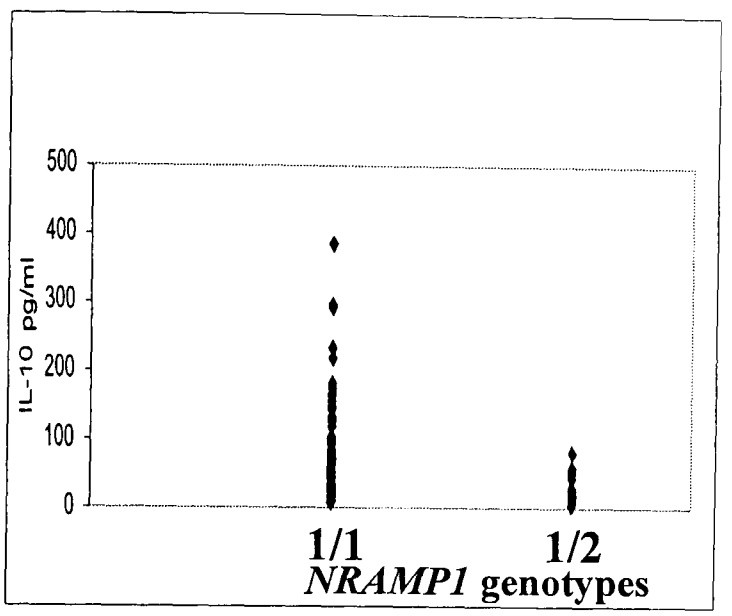
Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	0.47	2	0.78
IL-1β		0.32	2	0.85
Il-10		1.39	2	0.49
TNF	LAM&IFN-γ	0.98	2	0.6
IL-1β		0.20	2	0.9
IL-10		1.35	2	0.5
TNF	LPS	5.3	2	0.07
IL-1β		3.76	2	0.15
IL-10		1.58	2	0.45
TNF	LPS&IFNγ	3.5	2	0.17
IL-1β		2.76	2	0.25
IL-10		2.22	2	0.33

Table 5.3.1ii *NRAMP1* (274 C/T exon 3)

5.3.1iii *NRAMP1* (469+14 G/C intron 4)

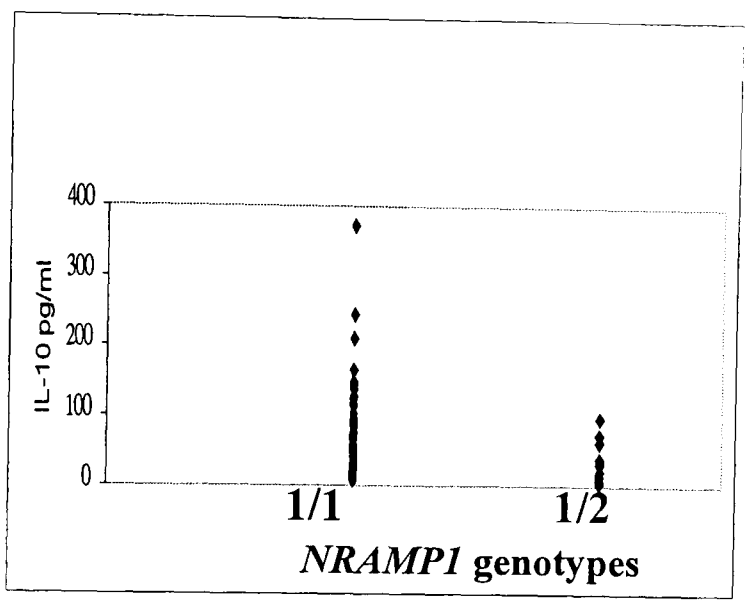
The genotype 2/2 was not observed. P valves were compared between two genotypes for each cytokine and stimulant.

Table 5.3.1iii shows that whole blood secretion of IL-10 after LAM stimulation was lower in NRAMP intron 4 1/2 genotypes (p=0.008), and IL-10 secretion was also lower after priming with IFN-γ and LAM stimulation (p=0.02). Alleles of NRAMP1 (469+ 14 G/C) were shown to influence LAM induced IL-10 secretion (p=0.008). Genotypes were still associated after priming with IFN-γ (p=0.02).



a) **LAM induced IL-10 and genotypes**

(p-value 0.0076)



b) **LAM and IFN-γ induced IL-10 and genotypes**

(p-value 0.02)

FIG. 5.4 *NRAMP1* INT4 genotypes and IL-10 secretion

Individuals were typed for the *NRAMP1* INT4 polymorphism. The 1/1 genotype represents homozygous for the *NRAMP1* INT 4 for the allele 1 and 1/2 heterozygote. 2/2 homozygous for the *NRAMP1* INT4 allele 2 was not observed. Cytokine responses to LAM alone and LAM and IFN-γ are shown in relation to host genotype. A) LAM induced IL-10 and *NRAMP1* INT4 genotypes b) IFN-γ enhancement of IL-10 and *NRAMP1* INT4 genotypes.

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	0.40	1	0.91
IL-1β		0.21	1	0.65
Il-10		7.13	1	0.008
TNF	LAM&IFN-γ	0.83	1	0.36
IL-1β		0.13	1	0.72
IL-10		5.48	1	0.02
TNF	LPS	0.03	1	0.872
IL-1β		0.00	1	1.0
IL-10		1.27	1	0.26
TNF	LPS&IFNγ	0.31	1	0.58
IL-1β		0.51	1	0.45
IL-10		1.86	1	0.17

Table 5.3.1iii *NRAMP1* (469+14 G/C intron 4)

5.3.1iv *NRAMP1* (1465-85 G/A.intron13)

In addition, for the *NRAMP1* intron 13 polymorphism table 5.3.1iv shows that whole blood secretion of IL-10 after LPS stimulation was lower in 1/2 genotype (P=0.04), whereas, IL-1β secretion after priming with IFN-γ and LPS stimulation was lower in the same genotypes (p=0.05).

P valves as compared between three genotypes for each cytokine and stimulant.

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	0.91	2	0.63
IL-1β		0.54	2	0.76
Il-10		1.37	2	0.50
TNF	LAM&IFN-γ	2.86	2	0.24
IL-1β		0.85	2	0.65
IL-10		1.5	2	0.48
TNF	LPS	1.8	2	0.41
IL-1β		2.8	2	0.25
IL-10		6.43	2	0.04
TNF	LPS&IFNγ	1.55	2	0.46
IL-1β		5.97	2	0.05
IL-10		2.6	2	0.27

Table 5.3.1iv *NRAMP1* (1465-85 G/A intron13)

5.3.2i *IL1RN*

The genotypes 1.1 and 1.2 were the most frequent in the population. The influence of genotype on LAM induced responses was only possible for these genotypes. The influence of genotype on LPS induced responses was possible for the 1.4 genotype. Whole blood secretion of IL-10 after stimulation with LPS alone or when pre incubated with IFNγ was lower in individuals with the *IL1RN* 2/2 genotype those with *IL1RN* 1/1 genotype (p=0.002 and 0.005 respectively). Also, LAM induced IL-10 was lower in the 2/2 genotypes (p=0.05). All other genotypes occurred at low paired observation frequency to allow for any meaningful statistical analysis.

P valves as compared between three genotypes for each cytokine and stimulant.

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	0.66	1	0.42
IL-1β		0.04	1	0.83
Il-10		3.59	1	0.05
TNF	LAM&IFN-γ	0.15	1	0.69
IL-1β		0.08	1	0.78
IL-10		1.25	1	0.26
TNF	LPS	1.48	2	0.48
IL-1β		0.58	2	0.75
IL-10		12.1	2	0.002
TNF	LPS&IFNγ	0.55	2	0.76
IL-1β		0.53	2	0.77
IL-10		19.5	2	0.005

Table 5.3.2i ***IL1RN***

5.3.2ii *IL1B* -511

P valves were compared between three genotypes for each cytokine and stimulant.

None of the alleles of this polymorphism was shown to be influencing cytokine-induced responses (Table 5.3.2ii).

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	1.25	2	0.54
IL-1β		2.1	2	0.35
Il-10		0.92	2	0.63
TNF	LAM&IFN-γ	2.6	2	0.27
IL-1β		3.0	2	0.22
IL-10		0.45	2	0.8
TNF	LPS	1.94	2	0.4
IL-1β		0.35	2	0.84
IL-10		0.45	2	0.79
TNF	LPS&IFNγ	4.4	2	0.11
IL-1β		2.3	2	0.31
IL-10		0.31	2	0.86

Table 5.3.2ii *IL1B* -511

5.3.2iii *IL1B* +3953

P valves were compared between three genotypes for each cytokine and stimulant.

None of the alleles of this polymorphism was shown to be influencing cytokine-induced responses (Table 5.3.2iii).

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	0.16	2	0.92
IL-1β		0.15	2	0.93
Il-10		1.85	2	0.4
TNF	LAM&IFN-γ	1.64	2	0.4
IL-1β		0.73	2	0.96
IL-10		0.53	2	0.76
TNF	LPS	4.04	2	0.13
IL-1β		0.62	2	0.73
IL-10		2.4	2	0.3
TNF	LPS&IFNγ	1.45	2	0.48
IL-1β		2.8	2	0.25
IL-10		1.4	2	0.50

Table 5.3.2iii *IL1B* +3953

5.3.3i *IL4*P2

P valves as compared between three genotypes for each cytokine and stimulant.

Whole blood secretion of TNF after priming with either with IFN-γ and LPS or with LPS alone was lower in *IL-4*P2 2/2 genotypes (p=0.03 and p=0.08).

Whole blood secretion of TNF after priming with IFN-γ and LPS or with LPS stimulation was significantly different between genotypes (p=0.004, p=0.013). Also stimulation with IFN-γ and LPS suppressed IL-10 production in *IL-4*P2 2/2 genotypes (p=0.04). Alleles of *IL-4*P2 were shown to influence LPS induced TNF secretion (p =0.08). The pre-incubation of IFN-γ further enhanced the influence of these alleles (p =0.03).

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	2.35	2	0.31
IL-1β		3.49	2	0.17
Il-10		2.92	2	0.23
TNF	LAM&IFN-γ	1.11	2	0.57
IL-1β		2.4	2	0.3
IL-10		2.4	2	0.3
TNF	LPS	4.95	2	0.08
IL-1β		3.35	2	0.19
IL-10		3.48	2	0.17
TNF	LPS&IFNγ	6.87	2	0.03
IL-1β		2.5	2	0.29
IL-10		0.99	2	0.03

Table 5.3.3i *IL4*P2

5.3.4i *TNFA* -863

P valves as compared between three genotypes for each cytokine and stimulant.

Comparison could not be made for LAM induced responses for the rarer genotype 2. Whole blood secretion of TNF after stimulation with IFN-γ and LPS or LPS alone differ between *TNFA* -863 genotypes (p=0.04, p=0.02 respectively) similarly secretion of IL-1β after stimulation with IFN-γ and LPS differed between groups (p=0.04). Alleles of TNF -863 were shown to influence LPS induced TNF (p =0.02) Priming with IFN-γ did not enhance this effect (p =0.04).

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	0.002	1	0.96
IL-1β		0.12	1	0.73
Il-10		0.05	1	0.82
TNF	LAM&IFN-γ	0.98	1	0.32
IL-1β		0.79	1	0.37
IL-10		0.13	1	0.72
TNF	LPS	7.67	2	0.02
IL-1β		4.22	2	0.12
IL-10		1.2	2	0.55
TNF	LPS&IFNγ	6.2	2	0.04
IL-1β		6.2	2	0.04
IL-10		3.17	2	0.21

Table 5.3.4i ***TNFA*** **-863**

5.3.4ii *TNFA* -857

P valves as compared between three genotypes for each cytokine and stimulant.

Comparison could not be made for LAM induced responses for the rarer genotype 2.2

None of the genotypes are alleles of this variant influenced cytokine response (Table 5.3.4ii).

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	0.013	1	0.91
IL-1β		0.05	1	0.82
Il-10		0.17	1	0.68
TNF	LAM&IFN-γ	0.35	1	0.55
IL-1β		0.006	1	0.94
IL-10		0.001	1	0.98
TNF	LPS	1.11	2	0.57
IL-1β		0.63	2	0.73
IL-10		1.86	2	0.4
TNF	LPS&IFNγ	1.16	2	0.56
IL-1β		0.93	2	0.63
IL-10		1.77	2	0.41

Table 5.3.4ii ***TNFA*** **-857**

5.3.4iii *TNFA*-376

P valves as compared between two genotypes for each cytokine and stimulant.

Homozygotes for the rarer genotype 2.2 were few and thus not possible to compare cytokine secretion by this allele.

Whole blood secretion of IL-1β after stimulation with IFN-γ and LAM differed between TNF A-376 genotypes (p=0.04) also secretion of IL-10 after stimulation with IFN-γ and LPS differed between groups (p=0.03). Alleles of *TNFA* –376 were shown to influence LPS induced IL-10 secretion (p =0.06). Pre-incubation with IFN-γ further enhanced the influence of these alleles (p = 0.03).

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	0.15	1	0.69
IL-1β		1.26	1	0.26
Il-10		0.65	1	0.42
TNF	LAM&IFN-γ	0.07	1	0.79
IL-1β		4.36	1	0.04
IL-10		1.8	1	0.18
TNF	LPS	1.62	1	0.2
IL-1β		1.46	1	0.23
IL-10		3.53	1	0.06
TNF	LPS&IFNγ	0.8	1	0.37
IL-1β		2.55	1	0.11
IL-10		4.86	1	0.03

Table 5.3.4iii *TNFA*-376

5.3.4iv *TNFA* -308

None of the alleles of the *TNFA*-308 polymorphism influenced induced TNF and IL-10 responses significantly (Table 5.3.4iv). Alleles of *TNFA*-308 did marginally influence IL-1β levels induced when cells were pretreated with rIFNγ before stimulation with LAM (0.07).

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	0.99	2	0.6
IL-1β		3.11	2	0.21
Il-10		1.56	2	0.45
TNF	LAM&IFN-γ	0.53	2	0.77
IL-1β		5.32	2	0.07
IL-10		2.6	2	0.27
TNF	LPS	2.6	2	0.27
IL-1β		1.5	2	0.47
IL-10		0.03	2	0.98
TNF	LPS&IFNγ	1.22	2	0.54
IL-1β		1.55	2	0.46
IL-10		0.83	2	0.66

P valves obtained after comparing between the three genotypes for each cytokine and stimulant.

Table 5.3.4iv *TNFA*-308

Figures 5.7 and 5.8 illustrating cytokine levels between *TNFA*-308 genotypes are on pages 165 and 166.

5.3.4v *TNFA* -238

p-valves as compared between three genotypes for each cytokine and stimulant whole blood secretion off IL-1β after stimulation with IFN-γ and LPS differed between TNF -238 genotypes (p=0.05).

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	1.27	2	0.5
IL-1β		3.11	2	0.2
Il-10		5.35	2	0.06
TNF	LAM&IFN-γ	3.07	2	0.22
IL-1β		3.72	2	0.16
IL-10		2.11	2	0.35
TNF	LPS	4.45	2	0.11
IL-1β		3.62	2	0.16
IL-10		3.93	2	0.14
TNF	LPS&IFNγ	1.54	2	0.46
IL-1β		6.1	2	0.05
IL-10		2.54	2	0.28

Table 5.3.4v *TNFA* -238

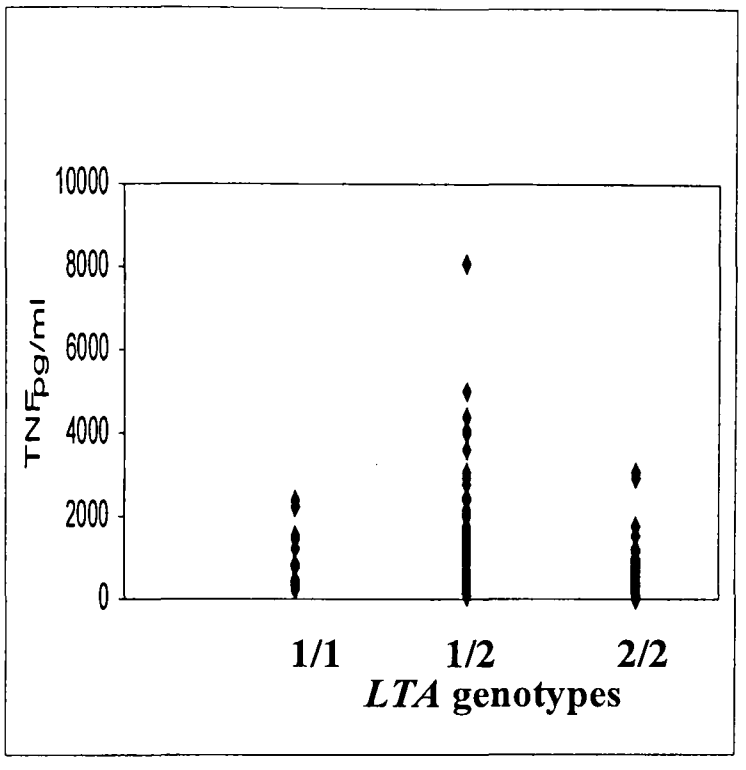
5.3.4viia Lymphotoxin alpha (*LTA*)

p-valves as compared between three genotypes for each cytokine and stimulant.

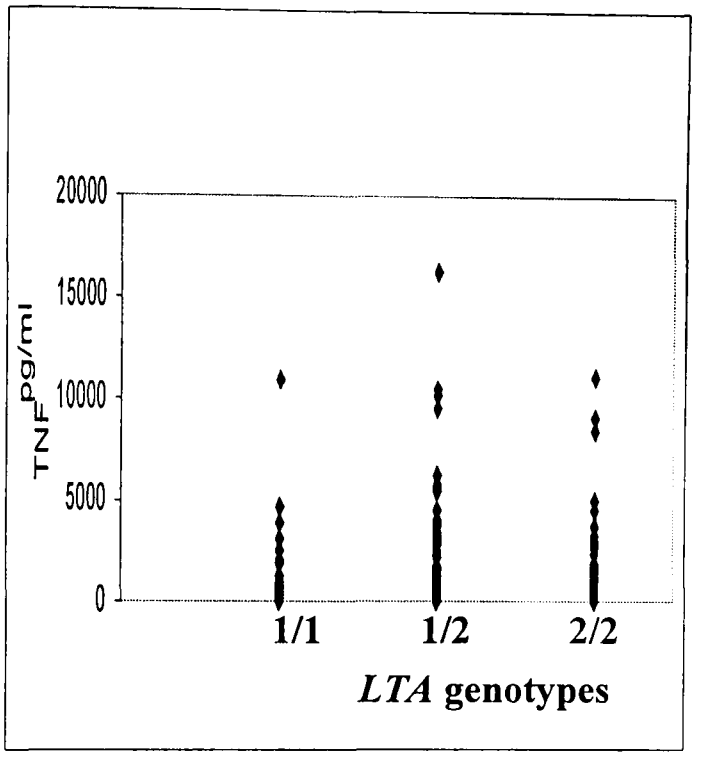
Comparison was made between three genotypes. Whole blood secretion of TNF and IL-1β upon stimulation by either LPS or LAM differs significantly between genotypes. IL-10 levels were not influenced by the genotypes of this polymorphism.

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	17.02	2	0.0002
IL-1β		5.75	2	0.05
Il-10		4.08	2	0.13
TNF	LAM&IFN-γ	11.8	2	0.003
IL-1β		5.4	2	0.07
IL-10		2.4	2	0.3
TNF	LPS	10.8	2	0.005
IL-1β		3.38	2	0.18
IL-10		4.11	2	0.13
TNF	LPS&IFNγ	9.46	2	0.009
IL-1β		2.75	2	0.25
IL-10		1.7	2	0.42

Table 5.3.4viia *LTA*



a) *LTA* NcoI genotypes and LAM induced TNF (p-value 0.0002)

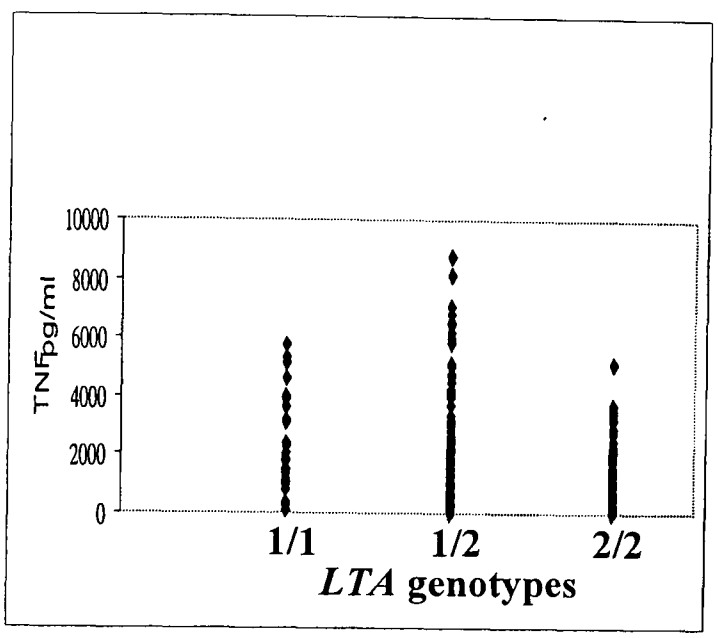


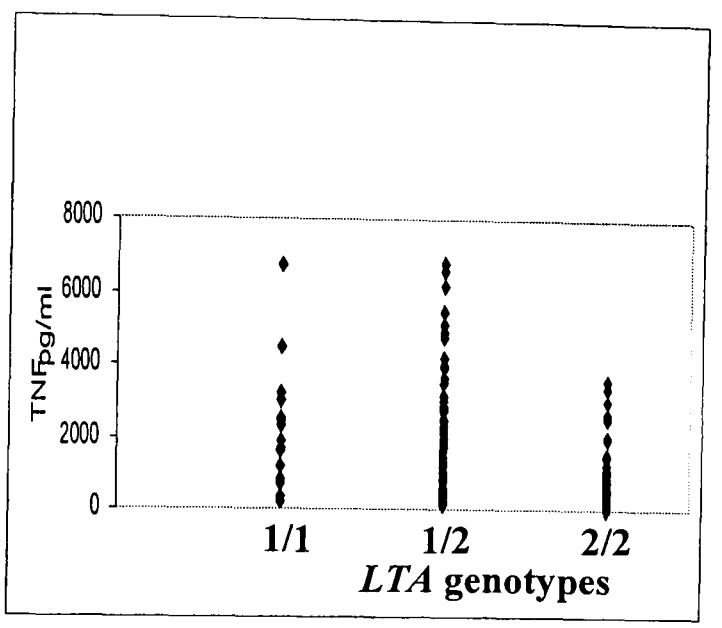
b) *LTA* NcoI genotypes and LPS induced TNF (p-value 0.005)

FIG. 5.5 *LTA* genotypes and TNF secretion

Individuals were typed for the *LTA* polymorphism and divided into three groups according to their genotypes. 1/1 represents homozygous for the *LTA 1* allele, 2/2 represents homozygous for the *LTA 2* allele and 1/2 represents heterozygotes. Cytokine responses to either a) LAM and b) LPS are shown in relation to host genotype. The p-value compares the difference between the three genotypes in their ability to induce cytokine responses.

Stimulant	MEAN TNF by genotype		
	1/1	1/2	2/2
LAM	940 (n=24)	1345 (n=72)	623 (n=48)
LAM+γ	2062	2205	1124
LPS	1037 (n=32)	1050 (n=115)	680 (n=87)
LPS+γ	1892	2067	1457





a) Effect of IFN -γ on LAM induced TNF (p-value 0.003)

b) Effect of IFN-γ on LPS induced TNF (p-value 0.005)

FIG. 5.6 *LTA* genotypes and effect of IFN-γ on TNF

TNF produced in response to either LAM or LPS after IFN-γ enhancement plotted according to host genotype. Individuals were typed for the *LTA* polymorphism and divided into three groups according to their genotypes. 1/1 represents homozygous for the *TNFA* allele, 2/2 represents homozygous for the *TNFA* 2 allele and 1/2 represents heterozygotes. Cytokine responses to either a) LAM and b) LPS are shown in relation to host genotype.

Secretion of these cytokines were generally higher in individuals with *LTA* 1/2 genotypes (p=0.0002 and p=0.05 for LAM induced TNF and IL1β). TNFβ 1/2 genotype demonstrated higher levels for LPS induction of TNF than those with *LTA* 2/2 genotypes did. Priming with IFN-γ, had no effect on either LAM or LPS induced secretion of the three cytokines. IL-10 secretion between the three genotypes was not significantly affected by either of the stimulants.

5.3.4viib ***LTA***

Because of the positive findings when genotypes of *LTA* were analysed (Table 5.3.4viia) comparisons were made between 1/1 and 2/2 homozygotes alone (Table 5.3.4viib). Whole blood secretion of TNF and IL-1β after stimulation with either LAM or LPS differed significantly between *LTA* genotypes. Secretion of these cytokines was generally higher in individuals with *LTA* 1/1 genotypes (p=0.0001 and p=0.02 for LAM induced TNF and IL1β). *LTA* 1/1 genotype demonstrated higher levels for LPS induction of TNF than those with *LTA* 2/2 genotypes.

Cytokines	**Stimulant**	χ^2	**DF**	**p-value**
TNF	LAM	16.43	1	0.0001
IL-1β		5.21	1	0.02
Il-10		3.93	1	0.05
TNF	LAM&IFN-γ	11.73	1	0.0006
IL-1β		4.69	1	0.03
IL-10		2.37	1	0.12
TNF	LPS	10.4	1	0.001
IL-1β		3.36	1	0.06
IL-10		4.09	1	0.04
TNF	LPS&IFNγ	8.96	1	0.003
IL-1β		2.7	1	0.1
IL-10		1.6	1	0.21

Table 5.3.4viib ***LTA***

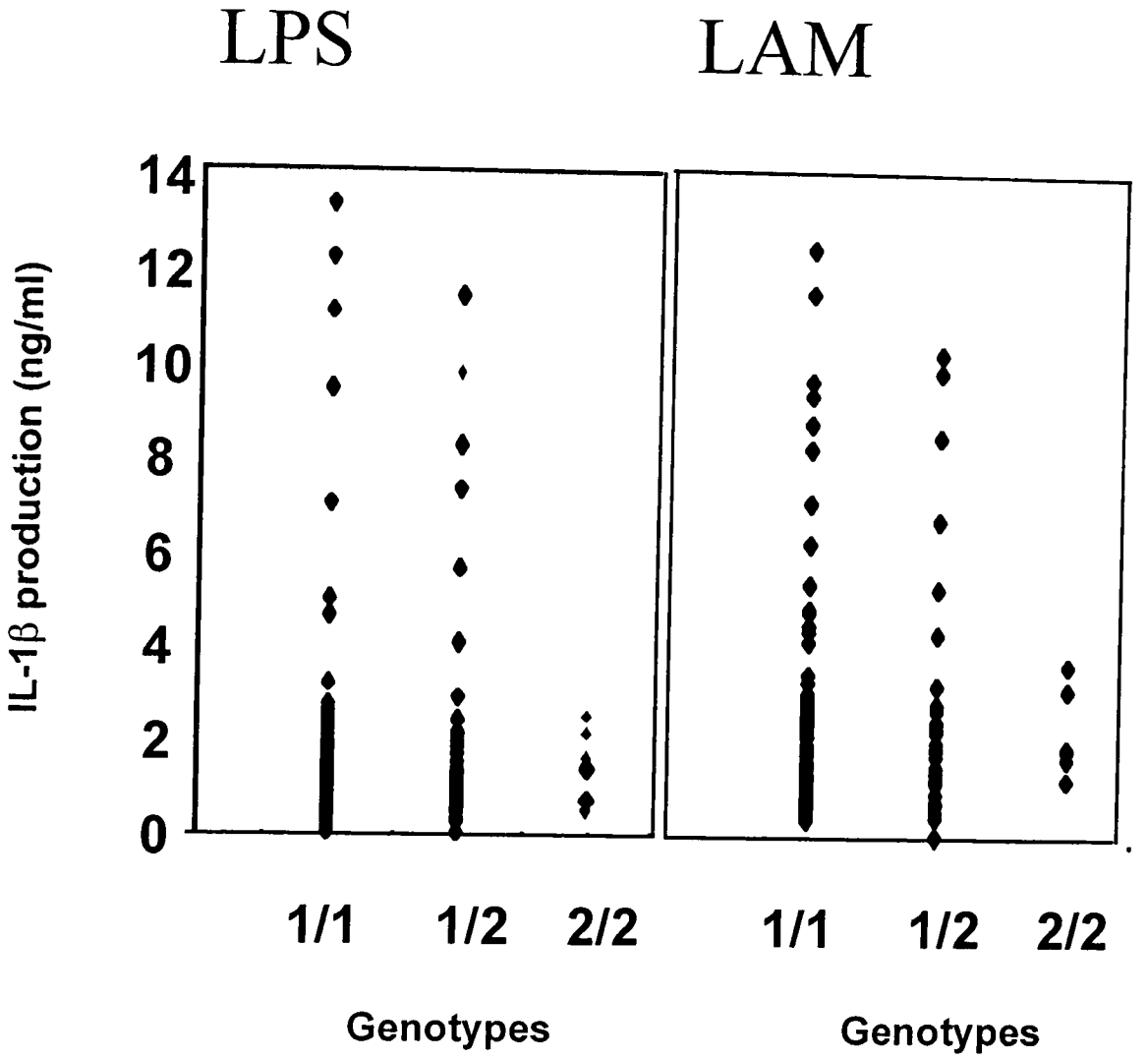


FIG. 5.7 *TNFA*-308bp genotypes and IL-1β secretion.

Figure 5.7 shows a scatter plot of *TNFA* -308 bp genotypes and IL-1β secretion. It demonstrates that the 2/2 homozygotes are low producers, 1/2 heterozygotes are intermediate producers, and the 1/1 homozygotes are high producers of IL-1β. However, due to the small number of 2/2 homozygotes there is no significant difference between the groups.

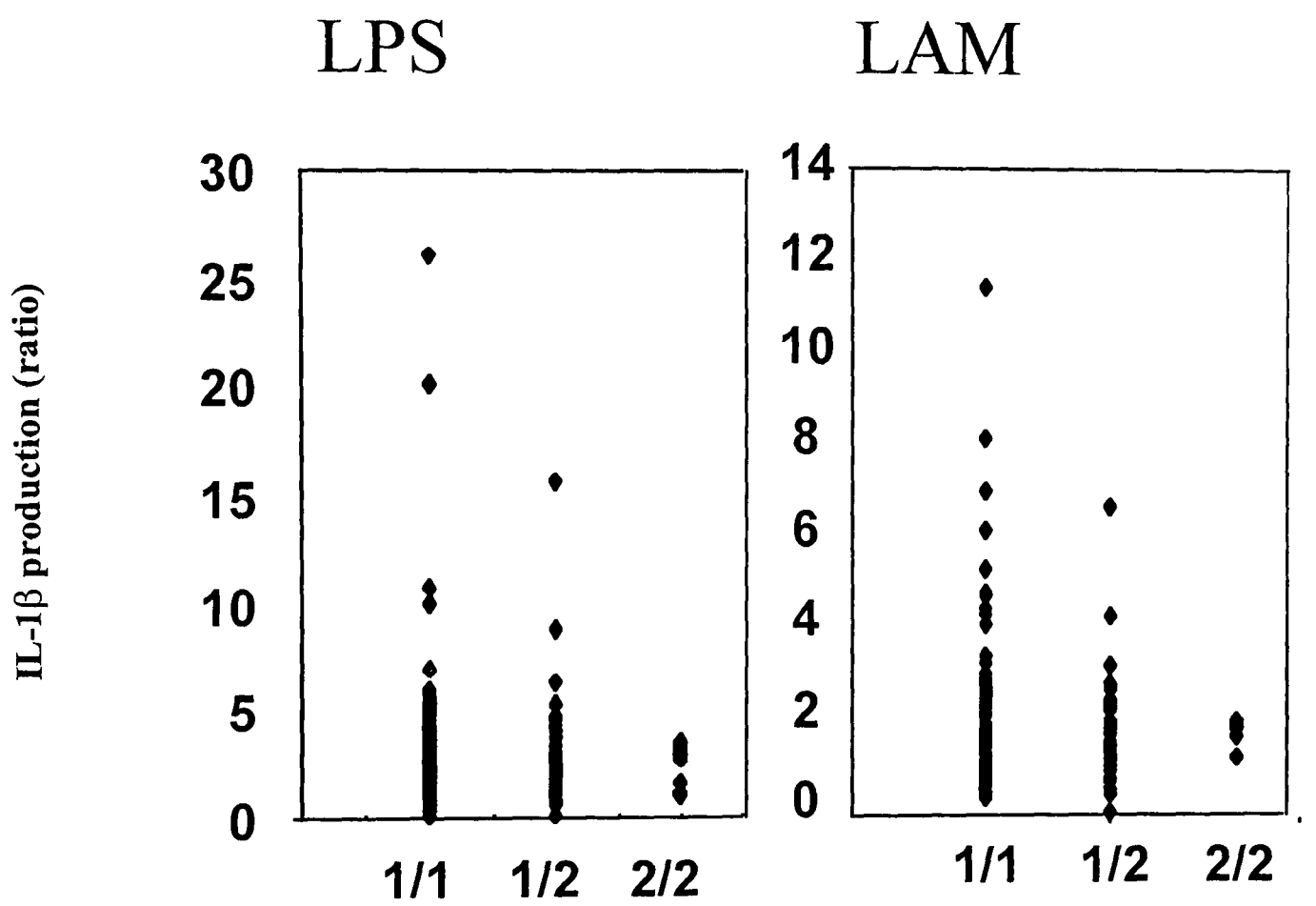


FIG. 5.8 *TNFA*-308bp genotypes and IFN-γ enhancement of IL-1β production.

Figure 5.8 shows the scatter plot of indices of IFN- γ up-regulation for both LPS and LAM induced IL-1β production. The trend was similar to that observed when LPS or LAM alone was used as stimulant and IFN-γ did not influence this trend.

P valves when compared between homozygote genotypes for each cytokine and stimulant. Priming with IFN-γ, had no effect on either LAM or LPS induced secretion of the three cytokines. IL-10 secretion between the three genotypes remained unaffected by either of the stimulants.

5.3.5i DdeI/TT *IFNGR1*

P valves as compared between three genotypes for each cytokine and stimulant. Homozygosity for this double deletion polymorphism is rare in the healthy population (0.01). This polymorphism is at the γ activated site (GAS) in the promoter of *IFNGR1*. A murine monocyte-like cell-line WEHI-3 was shown to be incapable of utilising GAS element present in the promoter of any γ inducible gene in response to IFNγ. There was no particular trend in the ability of alleles of this deletion to induce cytokine responses

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	0.69	2	0.7
IL-1β		0.21	2	0.9
Il-10		0.35	2	0.84
TNF	LAM&IFN-γ	1.87	2	0.4
IL-1β		0.05	2	0.97
IL-10		1.52	2	0.47
TNF	LPS	2.31	2	0.32
IL-1β		1.35	2	0.5
IL-10		1.16	2	0.56
TNF	LPS&IFNγ	0.67	2	0.71
IL-1β		1.45	2	0.48
IL-10		0.99	2	0.61

Table 5.3.5i DdeI/TT *IFNGR1*

In summary four SNPs were shown to influence cytokine responses. These were NRAMP1 469 polymorphism, *IL1RN*, *TNFA*-863, and *LTA*. IL-10 production was influenced by *NRAMP1* 469 polymorphism after LAM stimulation and *IL1RN* polymorphism after stimulation with both LAM and LPS. TNF production was influenced by *LTA* polymorphism after both LPS and LAM stimulation. IL-1β levels were influenced by *LTA* polymorphism after LPS alone. *TNFA* -863 genotypes influenced LPS induced TNF. This study showed that allelic variants of two genes (*TNFA*-863 and *LTA*), that encode pro inflammatory molecules influence pro inflammatory cytokine responses (TNF and or IL-1β) whereas, allelic variant of genes that encode anti inflammatory molecules (*IL1RN* and *NRAMP1*) influence anti inflammatory cytokine responses (IL-10). Table 5.4 below summaries these findings. An explanation for this may be that because *TNFA* and *LTA* are encoded on the short arm of chromosome six and alleles of both genes that are in linkage disequilibrium are influencing the same cytokines. Indeed, haplotype analysis of the TNF-region in table 6.3.5 reveals linkage disequilibrium between alleles of the *TNFA* -863 and *LTA*. The allele 2 (G) from previous studies and allele 1 of this study is in linkage disequilibrium with allele 1 (C) of the *TNFA* -863 polymorphism. Haplotype 4 consists of these variants and they are more in controls than cases. (Table 6.3.5) Interestingly, alleles of both genes were shown to be influencing measured TNF levels.

Table 5.4 SUMMARIES OF CANDIDATE GENES SHOWN TO INFLUENCE MICROBIAL INDUCED RESPONSES.

MARKER	STIMULANT	G	OBS	A.SUM	CYTOKINE	X^2	D.F	p-value
NRAMP1 (469+14)	LAM	1.1	112	67.2	IL-10	7.13	1	0.0076
		1.2	15	40				
	LAM+IFN	1.1	112	66.8	IL-10	5.48	1	0.019
		1.2	15	43.1				
LTA	LAM	1.1	17	72.4	TNF	17.0	2	0.0002
		1.2	72	80.7				
		2.2	48	50.3				
	LAM	1.1	18	66.6	IL-1β	5.75	2	0.05
		1.2	69	74.1				
		2.2	46	56.5				
	LAM+IFN	1.1	18	75.6	TNF	11.8	2	0.003
		1.2	72	78.6				
		2.2	48	53.5				
	LAM+IFN	1.1	18	65.9	IL-1β	5.4	2	0.067
		1.2	70	74.5				
		2.2	46	57.4				
	LPS	1.1	36	143.	TNFα	10.8	2	0.005
		1.2	124	135				
		2.2	90	106				
	LPS+IFN	1.1	36	144	TNF	9.46	2	0.009
		1.2	124	134				
		2.2	91	109				
TNFA-863	LPS	1.1	230	132	TNF	7.7	2	0.02
		1.2	26	128				
		2.2	3	11				
	LPS+IFN	1.1	231	131	TNF	6.2	2	0.04
		1.2	26	136				
		2.2	3	24				
IL1RN	LAM	1.1	111	59.9	IL-10	3.59	1	0.05
		1.2	12	80.5				
	LPS	1.1	111	95.1	IL-10	12.1	2	0.002
		2.2	1	21.5				
		1.2	15	144				
	LPS+IFN	1.1	180	95	IL-10	19.5	2	0.005
		2.2	1	53				
		1.2	15	142				

Table 5.4 shows only the markers, which genotypes differ in their ability to regulate quantitative cytokine measurements. G represents the genotype at a given locus. OBS represents numbers of observations per genotype for each marker. A.SUM is the average-ranksum and this is the ratio of rank sum and number of observations. The frequency of *IL1RN* 2.2 homozygotes was low.

5.4 DISCUSSION

Several single nucleotide polymorphisms SNPs have been analysed along with microsatellite markers. Most of the polymorphism shown to be influencing induced cytokine production in this study were SNPs that have been reported to have biological function. A single base change in DNA sequence can greatly increase or decrease binding of a particular transcription factor and affect protein production, (Knight *et al.,* 1999, Wilson *et al.,* 1997) whereas microsatellites are tandem repeats without any proven biological function. The tables for the microsatellite data are in appendix 5.

From results obtained it is easy to speculate that the *LTA NcoI* polymorphism may represent a functionally relevant gene variation. However, it is difficult to associate intronic polymorphisms with possible functional relevance. The *TNFB*1* allele possesses a triplet variation coding for asparagine at amino acid position 26, whereas the *TNFB* 2* allele has threonine at this position from previous workers (Messer *et al.,* 1991). This study reports the allele 1 from this study (10.5kb fragment) or *TNFB*2* homozygotes (from previous work) were associated with higher TNF production upon stimulation of whole blood system with LPS and LAM. In contrast, an earlier in-vitro study using PBL stimulated with PHA demonstrated higher TNFβ and not TNFα levels in *TNFB*1* homozygotes when compared with the *TNFB*2* homozygotes (Messer *et al.,* 1991). Studies by both Stuber *et al.,* 1999 and Pociot and co workers (1993) found *TNFB*2* the equivalent of allele 1 of this study to be associated with high TNF. This study also demonstrates lower IL-1β levels upon LAM stimulation of whole blood in *TNFB*1* homozygotes or allele 2. TNF levels were 25% higher in the *TNFB*1* homozygotes than the *TNFB*2* homozygotes judged by average ranksum. The average ranksum was 72.4

for the *TNFB*1* homozygote while *TNFB*2* homozygote was 50.3. In addition, IL-1β levels were generally 15 % higher in the *TNFB*1* homozygotes. The average ranksum for the *TNFB*1* homozygote was shown to be 66.6 while *TNFB*2* homozygote was 56.6. IL-10 levels were not affected by the *LTA* genotypes upon stimulation with either of the stimulants.

It may be that this marker is closely linked to another as yet undetected genomic variations. A new gene BAT1 has been cloned within the TNF gene cluster. There are indications that this gene is functionally important (Patricia Price unpublished).

Genotype 2/2 of *IL1RN* is reported to be functionally relevant and has been shown to induce higher levels of *IL1RN*. Induction of IL-10 an anti-inflammatory cytokine differed significantly between *IL1RN* genotypes in this study.

This study shows that the *NRAMP1* 469 +14 G/C variant influence LAM induced IL-10 secretion. An in-vitro study using reporter gene constructs had showed that alleles of the *NRAMP1* CA repeat differ in their ability to drive gene expression. This study does not show any difference in the ability of the alleles of that polymorphism to influence cytokine production using the wholeblood model. Rather, it is the *NRAMP1* intron 4 variant that was shown to be in linkage disequilibrium with the *NRAMP1* promoter allele 2 that was shown to be influencing IL-10 secretion.

This study was unable to demonstrate any influence of genotypes of the following macrophage candidate genes on microbial induced cytokine levels using the whole blood

model: - *NRAMP1* Z-DNA polymorphisms, *NRAMP1* 274 C/T, *IL9*, D17S250, IFNGR1, *IL1B* –511, *IL1B* +3953, TNFa, *TNFA*-308, *TNFA* -857 and ddelTT *IFNGR1* –470/471.

In conclusion, homozygotes for the *TNFB*2* allele 1 (G variant) differed in their ability to influence induced cytokine secretion by LPS and LAM. LPS and LAM induced TNF and IL-1β was significantly affected by LAM in these individuals. Priming with IFN-γ did not enhance this effect. The significant difference in the ability of alleles of this gene to influence pro inflammtory cytokine secretion was more pronounced for TNF, followed by IL-1β. Secretion of IL-10 was not affected. In addition, alleles of *NRAMP1* (469+14 G/C) transversion influenced LAM induced IL-10 secretion. This polymorphism was also significantly associated with TB in this population from a previous study (Bellamy *et al.*, 1998b). Allele 2 of this gene is in linkage disequilibrium with allele 2 of the *NRAMP1* CA Z-DNA polymorphisms previously shown to be poor at driving gene expression after LPS stimulation (Searle and Blackwell 1999). Alleles of anti-inflammatory IL-RA influenced both LAM and LPS induced anti-inflammatory IL-10 levels. IL-10 has been reported to induce higher levels of IL-1RA in people with either one or two copies of *IL1RN* allele 2 (Wilkinson *et al.*, 1999).

Differential regulation of genes in response to stimuli such as LAM or LPS may suggest unique roles for these stimulants during disease conditions. In chapter six the roles of particular alleles of these genes shown to be influencing cytokine induction was investigated for possible association with in disease trait TB.

CHAPTER 6

CASE CONTROL STUDY OF MACROPHAGE CANDIDATE GENES

6.1 Introduction

Immune responses to TB, being a complex disease, do not follow simple Mendelian monogenic inheritance patterns. Several genes are likely to be involved in the aetiology of disease. The innate mechanism is the first line of defence against mycobacteria and a central immunological requirement for a protective immune response in mycobacterial infections is the activation of macrophages for anti microbial activity and for the processing and presentation of mycobacterial antigens to specific T cells. Genes that influence macrophage activation, or are involved in the macrophage cascade are therefore good candidates for disease susceptibility to TB. Macrophage candidate gene selection in this study has been based on what is known from animal studies and human macrophage responses to mycobacterial infection. Published polymorphisms, having biological and functional relevance to macrophage activation have been selected. (Table 5.1).

Table 6.1 Macrophage candidate genes and relevance to TB.

Chromosome region	Relevance to TB	Reference
1q31-q32 (*IL10*)	IL-10 is a macrophage deactivator. IL-10 knockout mice exhibited increased antimycobacterial immunity. IL-10 suppresses immune responses in anergic TB patients. Higher IL-10 levels in recovered TB cases.	Murray and Young, 1999. Boussiotis *et al.*, 2000 Chapter 3, table 3.5.1 & 3.5.1
2q35 *NRAMP1*	1st single gene shown to control initial *M. bovis* infection in mice. NRAMP1 promoter CA repeat: -allele 3 protects against infectious diseases whereas allele 2 protects against autoimmunity.	Vidal *et al.*, 1995 Searle & Blackwell, 1999 Bellamy *et al.*, 1998b
2q 14 (*IL1B, IL1RN*)	IL-1RA is anti-inflammatory cytokine while IL-1β is pro inflammatory. Allele 2 of the 86bp VNTR is marginally associated TB in West Africans.	Dinarello, 1996 Bellamy *et al.*, 1998d
5q23-q32 *IL4* and *IL9*	Tuberculin responses were inversely related to atopy. IL-9 and IL-4 are linked to high total serum IgE levels. High total serum levels are hallmarks of asthma and atopy. In animal models, mycobacterial exposure prevents atopy.	Shirikawa *et al.*, 1997 Doull *et al.*, 1996 Erb *et al.*, 1998
6p21.3 HLA class III *TNFA* and *LTA*	TNF-308 A associated with lepromatous leprosy. TNF-376 OCT-1 binding genotype is found in 5% Africans with a 4-fold risk of increased susceptibility to cerebral malaria. TNF-238A is clinically associated with severe anaemic malaria. TNF-308 A is clinically associated with increased susceptibility to cerebral malaria and mucocutaenous leishmaniasis.	Roy *et al.*, 1997 Knight *et al.*, 1999 McGuire *et al.*, 1994 McGuire *et al.*, 1999 Cabrera *et al.*, 1995
6q23-24 (*IFNGR1*)	1st gene with fatal mutations shown to cause susceptibility to atypical mycobacterial infection in man.	Newport *et al.*, 1996 Jouanguy *et al.*, 1996
12q24 1 (*IFNG*)	IFN-γ is a potent macrophage activator. It is a Th1 cytokine and beneficial in TB. Disseminated tuberculosis in IFN-γ gene disrupted mice. Strong IFNγ response to mycobacteria is present in people that contain infection but there is a blunted response in those with active disease.	Flynn *et al.*, 1993 Cooper *et al.*, 1993 Ottenhoff *et al.*, 1998
17q11.2-q12 *NOS2A* *SCYA*	iNOS catalyses the production of free radical NO. NO works with TNF in an autocrine manner for enhanced killing of mycobacteria in murine macrophages. D17S250 marker is located closely to the genes that encode the small inducible chemokines. A locus on human chromosome 17 linked to TB in a family based genomic screening	MacMicking *et al.*, 1997 Lim, 2000

6.1.1 Interleukin 10 (*IL10*)

IL-10 plays an important role in the development of infectious disease. It was shown to affect macrophage responses during mycobacterial infections (Flesch *et al.,* 1994). Whole blood studies of monocyte function in chapter 3 show that recovered TB cases have higher levels of IL-10 when compared with these who had no disease (Table 3.5.1 & 3.5.2). High levels of IL-10 produced by T cells were associated with suppressed immune responses in anergic TB patients (Boussiotis *et al.,* 2000). High serum IL-10 levels in meningococcal sepsis was associated with poor or fatal outcome whereas the opposite was seen in patients with mild disease and good prognosis (Derkx *et al.,* 1995). Three different single nucleotide polymorphisms have been described in the *IL10* gene at nucleotide positions –1082, -819 and –592 (Turner *et al.,* 1997). In addition, two microsatellite repeats one 5' proximal at –1064bps of the TATA box (Eskdale & Gallagher, 1995) and another 5' distal (Eskdale *et al.,* 1996) to the initiation codon were reported. Genetic analysis of the human *IL10* promoter region placed the proximal dinucleotide repeat in a portion of the 5' untranslated region as defined by diminishing promoter function. (Kube *et al.,* 1995). This dinucleotide repeat has been associated with systemic lupus erythematosus (Eskdale *et al.,* 1997). The proximal *IL10* repeat was typed for allelic association with TB (Eskdale & Gallagher, 1995).

6.1.2 Natural resistance associated macrophage protein 1 (*NRAMP1*)

NRAMP1 is the human homologue of *Nramp1* , a gene originally identified as *Ity/ Lsh/Bcg* for its role in controlling *Salmonella typhimurium, Leishmania donovani, Mycobacterium bovis* infection in mice (Vidal *et al.,* 1995). Nramp1 is a metal ion transporter regulating and regulated by cellular iron levels (Searle and Blackwell, 1999). The precise mechanism of antimicrobial activity of NRAMP1 is still unclear.

However, in-vitro study of a functional *NRAMP1* 5' promoter polymorphism suggests that allele 3 may protect against infectious disease and allele 2 against autoimmune disease (Searle and Blackwell, 1999). Also, independent case control association (Gambian and Brazilian) studies demonstrated significant association between allele 2 and susceptibility to tuberculosis (Bellamy at al., 1998, Blackwell *et al.,* 1997). Allele 3 of this polymorphism has been associated with rheumatoid arthritis (Shaw *et al.,* 1996), juvenile rheumatoid arthritis (Sanjeevi *et al.,* 1999) and in diabetes patients with a first or second degree relative with rheumatoid arthritis (Esposito *et al.,* 1998). Four *NRAMP1* polymorphisms were typed. They are the functional promoter polymorphism containing the Z-DNA t (gt) 5ac(gt) 5ac(gt) ng or t (gt) 5ac(gt) ng repeats in the immediate 5' of the gene (Searle and Blackwell, 1999), a single nucleotide transition of C to T at nucleotide position 274 in codon 66 (Liu *et al.,* 1995), a single nucleotide transversion of G to C in intron 4 (Liu *et al.,* 1995), and another single nucleotide transition of G to A in intron 13 (Liu *et al.,* 1995).

6.1.3 Interleukin-1 beta (*IL1B*) & Interleukin-1 receptor antagonist *(IL-1RN)*

Interleukin-1β is involved in regulation of immunological and inflammatory reactions and has been implicated as a mediator of tissue destruction (Dinarello, 1991). IL-1β exerts its effects by interaction with the IL-1 receptor (IL-1R) and is antagonised by IL-1RA. The IL-1α, IL-1β, IL-1RA and IL-1R genes are located on a cluster on chromosome 2q (Steinkasserer *et al.,* 1992, Nicklin *et al.,* 1994). Intron 2 of IL-1RA contains a minisatellite (Tarlow *et al.,* 1993). IL-1β contains a single base transition at position –511 (Di Giovine *et al.,* 1992) and another at +3953 in exon 5 (Pociot *et al.,* 1992). The IL-1RA minisatellite is a pentallelic 86bp repeat sequence and allele two contains a potential transcription factor binding site for IFN-silencer A and IFN-silencer B (Tarlow *et al.,* 1993, Blakemore *et al.,* 1994). The pro-inflammatory

haplotype *IL1RN* A2- / *IL1B* +3953 A1+ is reported to be more common in patients with pleural tuberculosis and less common in patients with extrapulmonary disease. In addition, this haplotype is associated with reduced Mantoux response (Wilkinson *et al.,* 1999). These observations suggest that the *IL1RN* 2 allele is biologically relevant in TB. The *IL1B* -511 and +3953 polymorphisms were genotyped. The *IL1RN* 86 bp minisatellite was genotyped.

6.1.4 Th2 Cytokine cluster

Mycobacterial antigens such as purified protein derivatives of tuberculin (PPD) induce a Th1 response, whereas parasite antigens such as Toxocara excretory substances induce a Th2 response (Del Prete *et al.,* 1991). Th2 response is deleterious for TB; even a small Th2 component exacerbates the infection in mice (Rook & Hernandez-Pando, 1996). Given that Th2 cytokines inhibit Th1 response, individuals mounting prominent Th2 responses may be more susceptible to *M. tuberculosis*. It may be that Th2 immune dominance induced by intestinal parasites enhances susceptibility to tuberculosis (Bentwich *et al.,* 1999). Two genes within this cytokine cluster were typed for allelic association with TB. These are *IL4* and *IL9* (Marsh *et al.,* 1994). Two polymorphisms were genotyped in *IL4,* the intron 2, 70bp repeat and the intron 3 microsatellite GT repeat (Mout *et al.,* 1991). For *IL9*, a microsatellite repeat in intron 4 (Polymeropoulou *et al.,* 1991) was genotyped. Polymorphism in *IL9* was reported to be associated with serum Ig E levels (Doull *et al.,* 1996). The 70bp VNTR in *IL4* intron 2 has been associated with multiple sclerosis (Vandenbroeck *et al.,* 1997) but was not associated with myasthenia gravis (Huang *et al.,* 1998). There are reports of associations of allelic forms with bronchial hyper responsiveness in mice (Nicolaides *et al.,* 1997) and one report of an allelic association of allele 118 of the human IL-9 microsatellite and atopic condition (Doull *et al.,* 1996).

6.1.5 Tumour necrosis factor (*TNFA*)

TNF plays a major role in killing intracellular pathogens and much of the pathology of tuberculosis, including fever and weight loss is mediated via TNF. TNF concentration in different forms of malaria has been reported to correlate with disease severity (Kwiatkowski *et al.,* 1990). Thus, the amount of TNF produced may be critical in determining the outcome following infection with mycobacteria (Tracey, 1995). The *TNFA* gene is located in the MHC class III region and is polymorphic (Nedospasov *et al.,* 1991). Despite, contrasting in-vitro experimental observations on the functional importance of *TNFA* promoter polymorphisms on gene transcription there are increasing reports of *TNFA* allelic association with clinical diseases. The *TNFA*-238 polymorphism has been associated with severe malarial anaemia (Mc Guire *et al.,* 1999), chronic hepatitis B (Hohler *et al.,* 1998a), chronic active hepatitis C (Hohler *et al.,* 1998b) and psoriasis (Kaluza *et al.,* 2000). There are no associations with, scarring trachoma (Conway *et al.,* 1997) and early onset psoriasis (Jacob *et al.,* 1999). The *TNFA* –308 polymorphism has been associated with cerebral malaria (McGuire *et al.,* 1994), rheumatoid arthritis (Vinasco *et al.,* 1997), mucocutaneous leishmaniaisis (Cabrera *et al.,* 1995) scarring trachoma (Conway *et al.,* 1997) lepromatous leprosy (Roy *et al.,* 1997) and both forms of leprosy (Blackwell unpublished). Five *TNFA* promoter polymorphisms described at positions –238, (D'Alfonso & Riciardi, 1994), -308 (Wilson *et al.,* 1993), -376 (Hamann *et al.,* 1995), -857 and -863 (Uglialoro *et al.,* 1998) within the promoter of the *TNFA* gene were genotyped in this study. were genotyped. In addition an *NcoI* polymorphism in intron 2 of the lymphotoxin gene was genotyped.

6.1.6 Interferon gamma receptor alpha (*IFNGR1*)

A microsatellite FA1 within intron six of the *IFNGR1* gene was genetically mapped between the microsatellites D6S292 and D6S1699 (Altare *et al.,* 1998b). Intra-familial genotyping of an affected child was shown to have a unique pattern for FA1 when compared with her other four healthy siblings suggesting an autosomal recessive IFNγR1 deficiency (Altare *et al.,* 1998b). It was determined if particular alleles of this FAI repeat are associated with TB in our Gambian samples. In addition, the newly detected promoter deletion polymorphism at -470/471 of the *IFNGR1* was genotyped (section 4.3.3).

6.1.7 Interferon gamma (*IFNG*)

Interferon gamma is a Th1 cytokine with pleiotropic effects and has a beneficial role in immune responses to tuberculosis. Individuals with strong IFN-γ response to mycobacteria are able to efficiently contain the infection whereas those individuals with active disease have blunted response (Ottenhoff *et al.,* 1998). The intron 1 CA repeat has been associated with renal transplant rejection (Asderakis *et al.,* 1998) and lung allograft fibrosis (Awad *et al.,* 1998). Increased frequency of allele 5 and decreased frequency of allele 2 has been associated with Grave's disease (Siegmund *et al.,* 1993). This intron 1 CA repeat was genotyped for allelic association with TB in this study.

6.1.8 Inducible nitric oxide synthase (*NOS2A*) and small inducible chemokines

The gene that encodes the human iNOS (*NOS2A*) in maps to chromosome 17q11.2-q12 (Marsden *et al.,* 1994). From mice studies there is compelling evidence from both in vitro and in vivo studies that macrophages can kill various mycobacterial species including *M. tuberculosis* through NO dependent pathway (MacMicking *et al.,* 1997). In humans, the role of NO generated by the induction of nitric oxide synthase in the

killing of *M. tuberculosis* is controversial. Nicholson *et al.,* (1996) observed that pulmonary alveolar macrophages from patients with tuberculosis express inducible nitric oxide synthase (*NOS2A*) but thus far, there has been no evidence that human macrophages utilise NO to kill *M. tuberculosis*, although killing of other mycobacterial species has been described. Perhaps, the only data linking a role for NO generated by iNOS in TB is that reported by Rockett *et al.,* (1998). It was demonstrated that 1,25-D3 acts to suppress the growth of *M. tuberculosis* in a macrophage–like HL-60 line, through a NO dependent mechanism. Several polymorphisms have been described within the promoter of human *NOS2A* gene. These include a bi- allelic AAAT/AAAAT repeat element that occurs at frequencies of 0.84 and 0.16 among West Africans (Bellamy and Hill, 1997), and ten single nucleotide polymorphisms spanning about 2.5kb, 5'of the initiation codon (Burgner, 1999). A promoter polymorphism was weakly associated with severe malaria in Gabon (Kun *et al.,* 1998). I have analysed the allelic distribution of a functional polymorphic pentanucleotide repeat (CCTTT)n within the 2.5kb 5' upstream promoter region of the *NOS2A* gene in this study (Xu *et al.,* 1996.). Also, on the same chromosome, the microsatellite marker D17S250 (Weber *et al.,* 1990) linked to the small inducible cytokine gene cluster have been selected and typed in this study. D17S250 is in close proximity to the small inducible chemokine genes.

6.2 Genotyping of macrophage candidate genes

Genomic DNA was extracted from venous blood as described in chapter two from three hundred and twelve subjects studied in chapter 3 and 5 and two hundred and seventy-six TB cases. This was used as template for the PCR based methods as

described in 5.2. The polymorphisms typed are the same as chapter 5 with the exception of a RFLP in *IL9*.

6.2.1 RFLP in *IL9*

A RFLP in exon 5 of the *IL9* gene results in an amino acid substitution of methionine for threonine at codon 113 because of the nucleotide substitution of thymine for cytosine (Laitinen et al.,1997). The primers 5' ACT CTG GCT CTT GGC AGG TA 3' and 5' CCT ATG AGC CTG AGG GTC TG 3' were used to amplify exon 5 of the gene, which is 462 bp long. The polymorphism creates an *NcoI* site and this enzyme cuts the PCR fragment to 141 + 321 bp (variant allele). The PCR buffer number 1 (appendix 1) was used. The PCR conditions were 96° C denaturation for 5 minutes, followed by 30 cycles of another denaturation at 96° C for 30 seconds, then annealing at 65° C for 30seconds, elongation at 72°C for 30 seconds and a final extension for 5 minutes at 72°C. A mixture of 1µl of enzyme buffer and 0.5µl (5 units) of *NcoI* was added to 8.5µl of PCR product and digested for 6 hours at 37 degrees. The digested product was run on a 1% agarose gel containing ethidium bromide and visualised with UV light and photograph.

6.2.2 Data analysis

To address the problem of multiple testing, the Bonferroni adjustment was done and this involves assigning a cut-off based on the formula below. The cut off was done only when χ^2 test gave a significant p-value of = / < 0.05 for influence alleles on TB.

$$\text{Bonferroni cut off} = 0.05 / n$$

where 0.05 is an alpha level of 5% and n = number of alleles. If the observed p-value falls below this cut-off then the association is accepted as truly significant.

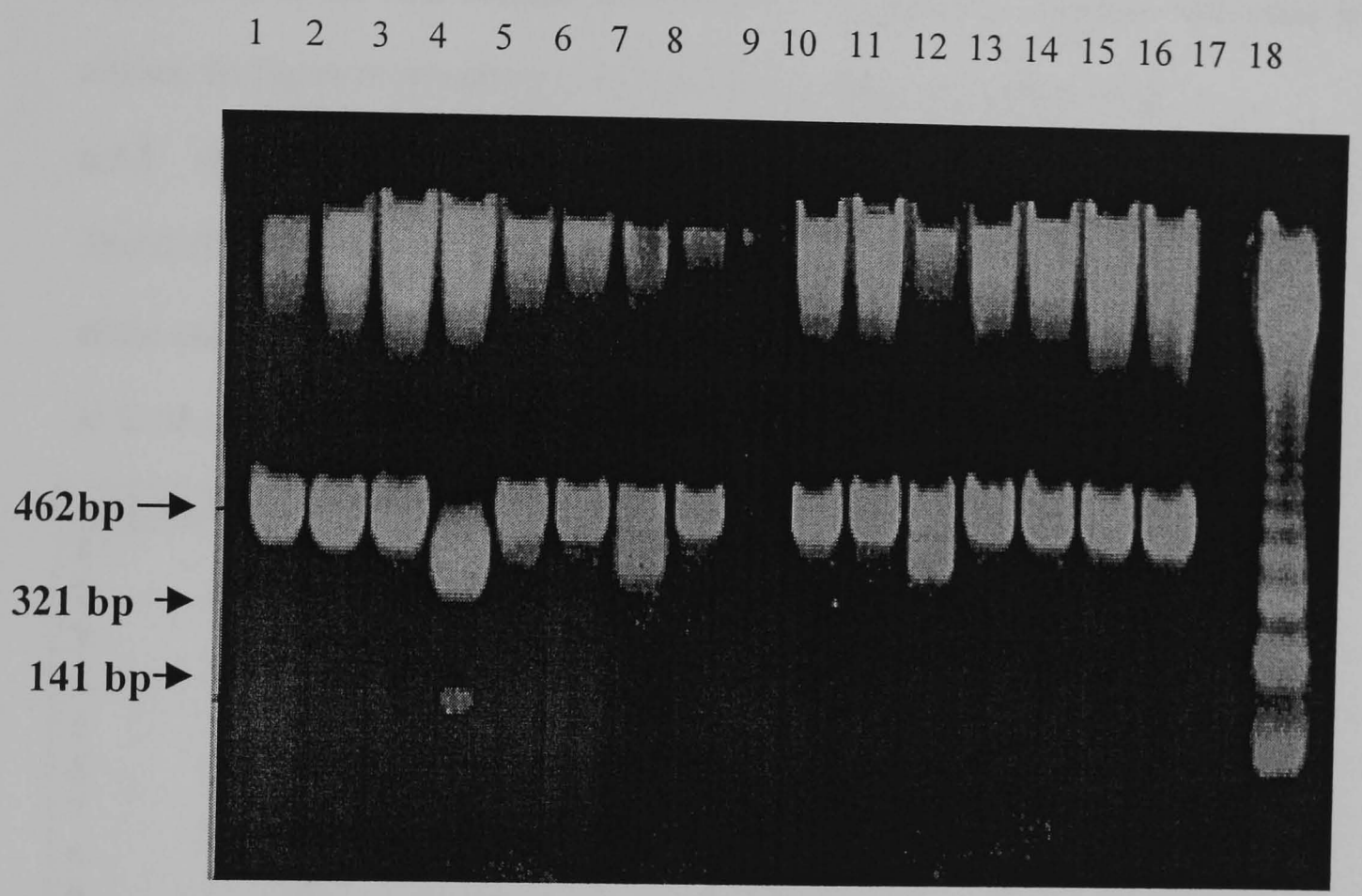


FIG. 6.1 Analysis of *IL9* T113M polymorphism

Figure 6.1 shows a RFLP of the *IL9* SNP at amino acid position 113 in exon 5, a substitution of threonine for methionine. The expected PCR product is 462 bp with an NcoI digestion site that gives 141 bp and 321 bp. Homozygote for allele 1 should have only the 321 bp and the 141 bp. Heterozygote should have all three bands.

Lane 18 is the molecular weight marker. lane 17 is a negative control, lanes 1, 2, 3, 5 & 6 are homozygotes for allele 2 , lane 4 is a homozygote for allele 1, and lanes 7 and 12 are heterozygotes.

6.3 Results: Influence of candidate genes on TB

Results were reported according to chromosome locations of markers studied and not in order of importance or significant association with TB. The allele number is represented in the first column followed by the number of controls and cases per alleles, the figure in parentheses are allele frequencies for a given allele.

6.3.1 *IL10*

The *IL10* proximal promoter repeat was genotyped. Eleven alleles were observed but allele one was not observed in the panel studied. There was no significant difference in *IL10* microsatellite alleles between controls and cases.

ALLELES	CONTROLS		CASES	
1	0		0	
2	4	(0.007)	2	(0.003)
3	32	(0.003)	5	(0.008)
4	36	(0.059)	50	(0.075)
5	52	(0.085)	72	(0.108)
6	178	(0.29)	177	(0.266)
7	100	(0.164)	93	(0.139)
8	122	(0.2)	115	(0.173)
9	47	(0.079)	79	(0.119)
10	48	(0.079)	44	(0.066)
11	19	(0.031)	27	(0.04)
12	2	(0.003)	2	(0.003)
Total	610		666	

Chi square χ^2 15.18 DF 10 p-value 0.125 NS Heterozygosity=0.819.

Table 6.3 1i *IL10*

6.3.2i *NRAMP1* 5' promoter repeats

All four reported alleles of the Z- DNA polymorphisms described were observed in this population. Analysis was done by both genotype-wise and allele wise method. Seven genotypes were observed. The homozygote 2/2 occurred more in cases but was not significantly different from the control (p=0.6). The frequency of this genotype was low at 0.026 in controls and its effect on TB was not obvious. The frequency of heterozygote 2/3 was moderately different between the two groups (p=0.06). The

homozygote 3/3 occurred more in controls and was significantly different between the two groups (p=0.01). Other alleles occurred at very low frequency to allow for any statistical analysis. Based on allele-wise method, allele two was observed more in cases and differed significantly in frequency from controls (p=0.03). Allele 3 was significantly more frequent in controls than in cases (p=0.01). Using allele wise method there was an overall significant difference between alleles (χ^2 10.36, DF2, p=0.016), which was not significant after Bonferroni adjustment (0.0125).

GENOTYPE	CONTROLS	CASES	χ^2	p-value	Odds ratio
1:1	0 (0)	1 (0.004)			
1:2	1 (0.004)	0 (0)			
1:3	14 (0.04)	11 (0.046)			
2:2	8 (0.026)	17 (0.06)	0.48	0.49	1.54
2:3	68 (0.22)	81 (0.29)	3.98	0.046	1.38
3:3	221 (0.71)	171 (0.6)	6.29	0.01	1.57
4:4	0 (0)	1			
Total	312	282			

Chi square χ^2 8.8 DF 5 p-value 0.1 NS Heterozygosity=0.277

Table 6.3.2ia *NRAMP1* 5' promoter repeats by genotypes

ALLELES	CONTROLS	CASES	χ^2	p-value	Odds ratio
1	15 (0.02)	19 (0.03)	0.71	0.4	0.7
2	88 (0.14)	104 (0.184)	4.68	0.03	0.7
3	524 (0.84)	439 (0.783)	6.06	0.01	1.46
4	0 (0)	2 (0.003)			
Total	624	564			

Chi square χ^2 10.36 DF 3 and p-valve **0.016**

Table 6.3.2ib *NRAMP1* 5' promoter repeats by alleles

ALLELES	CONTROLS	CASES	χ^2	p-value	Odds ratio
3:3	221 (0.71)	170 (0.61)	6.29	0.012	0.64
3:X	82 (0.26)	98 (0.35)	4.9	0.026	1.51.
X:X	9 (0.03)	12 (0.04)	0.49	0.49	1.51
Total	312	280			

Chi-square χ^2 6.79 DF 2 p-value **0.03**

Table 6.3.2ic *NRAMP1* 5' promoter repeats by contribution of allele 3

6.3.2ii *NRAMP1* (274 C/T)

For the *NRAMP1* 274 C to T transition polymorphism, the frequency of alleles did not differ significantly between controls and cases.

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
1:1 (TT)	5 (0.02)	5(0.03)		1 (T)	92 (0.16)	66(0.18)
1:2 (TC)	82(0.29)	56(0.3)		2 (C)	478 (0.84)	312(0.82)
2:2 (CC)	198(0.69)	128(0.67)				
Total	285	189		Total	570	378

Chi square by genotypes χ^2 0.51 DF 2 p-value **0.77,** Chi square by alleles χ^2 1.1, DF 1 p-value 0.3, Odds ratio 1.1, NS Heterozygosity=0.268

Table 6.3.2ii *NRAMP1* (274 C/T) by genotypes and alleles

6.3.2iii *NRAMP1* (469+14G/C)

The distribution of alleles of the *NRAMP1* 469+14 G to C transversion differed significantly. The homozygotes mutant condition was not observed. The frequency of the heterozygotes was more in the controls (0.35) than in the cases (0.16). This contrasts with an earlier study of TB cases and controls in The Gambia. That study observed that the frequency of the heterozygous was more in cases than controls (Bellamy *et al.,* 1998b). There are several similarities between the Bellamy study and this one; the studies are case control association studies of active TB cases, samples are from the same population, and both studies were corrected for ethnic admixture by stratification using the Mantel Haenzel test (M-H). B. The only differences are in the sample sizes, which is much larger in the Bellamy study and the use of dot blot hybridisation method versus RFLP. In any case it may be that this polymorphism though within an intron may represent truly functional gene polymorphism. This study also shows that alleles of this variant influence LAM induced IL-10 significantly (Table 5.4).

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
1:1 (GG)	178 (0.65)	141 (0.84)		1 (G)	453 (0.82)	309 (0.92)
1:2 (GC)	97 (0.35)	27 (0.16)		2 (C)	97 (0.18)	27 (0.08)
2:2 (CC)	0 (0)	0 (0)				
Total	275	168		Total	550	336

Chi-square by genotypes χ^2 19.94 DF 2 p-value **0.000013,** Chi square by χ^2 15.93 DF 1 p-value 0.00006 Odds Ratio 2.5 NS Hetrozygosity=0.113.

Table 6.3.2iii NRAMP (469+G/C) by genotypes and alleles

NRAMP1 **(1465-85G/A)**

The distribution of alleles for the intron 13 transition of 1465-85 G to A transition did not differ significantly between the two groups.

The distribution of alleles were the same in both controls and cases.

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
1:1 (AA)	45 (0.16)	38 (0.18)		1 (AA)	311 (0.57)	236(0.57)
1:2 (AG)	221 (0.81)	160 (0.78)		2 (GG)	237 (0.43)	170(0.43)
2:2 (GG)	8 (0.03)	8 (0.04)				
Total	273	206		Total	548	412

Chi-square by genotypes χ^2 0.74 DF 2 p-value 0.69, Chi square by alleles χ^2 0.03 DF 1 p - value 0.92. Odds ratio 1.02 NS Heterozygosity=0.49.

Table 6.3.2iv *NRAMP1* (1465-85G/A) by genotypes and alleles.

6.3.2v Haplotype analysis of *NRAMP1* polymorphisms

Haplotyping enhances the power of a study to detect an association, as functional alleles are likely to be in linkage disequilibrium with polymorphic markers typed. Unless molecular haplotyping methods are used, linkage phase is not known for individuals in a case control study. However, phase may be inferred using a likelihood approach based on the allele frequencies at each locus tested to compile the haplotype. Dr Frank Dudbridge has developed a computer programme which calculates haplotype frequencies within a population. This programme was used to analyse data for 3 *NRAMP1* polymorphisms and *IL9* polymorphisms (see section 6.3.4). Haplotypes were recorded in the following order: The *NRAMP1* (CA Z DNA polymorphism) *NRAMP1* 274 C/T and lastly the *NRAMP1* 1465 G/A variant.

Haplotype	Controls	Cases
3-2-1	0.38	0.29
3-2-2	0.38	0.31
2-2-1	0.05	0.16*
3-1-1	0.05	0.09
2-1-1	0.06	2.55e-11
2-2-2	0.02	0.045
1-2-1	1.3e-7	0.01
1-1-1	0.07	0.01
3-1-2	0.02	0.07
2-1-2	6.5e-7	3.4e-10
1-2-2	0.003	1.1e-4
4-2-1	0	0.003
4-2-2	0	0.003
1-1-2	0.0001	1.9e-12

Chi-square. χ^2 61.3 DF 15 p=1.5e-7 * = Haplotype 2-2-1 occurs more in cases than in controls.

Table 6.3.2v Haplotype analysis of *NRAMP1* variants

6.3.3i *IL1RN*

Four alleles were observed and the band sizes are listed in appendix 2. *IL1RN* allele frequencies were not significantly different between cases and controls (χ^2 5.05 DF 3, p=0.16). Allele 2 had been associated with increased levels of IL-1RA and increased risk of autoimmune diseases (Mansfield *et al.,* 1994, Clay *et al.,* 1994, and Blakemore *et al.,* 1994, 1995). The frequency of allele 2 differed between groups but was not statistically significant as the numbers were low, being more frequent in controls (0.04) than cases (0.024). Alleles 3 and 4 were observed at too low a frequency in this population to allow for any significant testing. There was no significant difference in the overall frequency.

GENOTYPES	CONTROLS	CASES		ALLELES	CONTROLS	CASES
1:1	209 (0.88)	162 (0.89)		1	447 (0.94)	343 (0.95)
1:2	17 (0.07)	9 (0.05)		2	17 (0.04)	9 (0.024)
1:3	0 (0)	3 (0.02)		3	0 (0)	3 (0.007)
1:4	12 (0.05)	7 (0.04)		4	12 (0.02)	7 (0.019)
Total	238	181		Total	476	362

Chi square result for allele frequency is χ^2 5.05 DF 3 p-value 0.16 NS. Heterozygosity =0.12

Table 6.3.3i *IL1RN*

6.3.3ii *IL1B* -511

Distribution of alleles of the *IL1B* –511 differed significantly between the two groups (p-value=0.002). The homozygote for the wild-type genotype (0.365) was twice as frequent as the homozygote mutant genotype (0.185) in TB cases whereas in the controls homozygous wild-type (0.24) and mutant (0.25) distribution was even. The mutant allele was less frequently seen in TB cases (0.41) than in controls (0.51).

The frequency of the wild type allele (T) in cases is 0.59 while the frequency of mutant allele (C) in cases is 0.41. This study shows that this polymorphism is significantly associated with TB in this population (genotype p-value=0.0045 and allele p-value 0.002).

GENOTYPE	CONTROLS	CASES	χ^2	p-value	Odds ratio
1.1 TT	67 (0.24)	85 (0.365)	10.7	0.001	1.93
1.2 TC	146 (0.51)	105 (0.45)	1.82	0.18	0.78
2.2 CC	71 (0.25)	43 (0.185)	2.82	0.09	0.68
Total	283	233			

Chi square analysis is χ^2, 10.78, DF 2,p-value 0.0045 for genotype wise analysis.

Table 6.3.3iia ***IL1β*-511 by genotypes**

ALLELE	CONTROLS	CASES	χ^2	p-value	Odds ratio
1 T	280 (0.49)	275 (0.59)	3.142	0.76	0.812
2 C	288 (0.51)	191 (0.41)			
Total	568	466			

Chi square analysis is χ^2 9.33 DF 1 p-value 0.002 Odds ratio 1.48 S Heterozygosity =0.5

Table 6.3.3iib ***IL1β*-511 by alleles**

6.3.3iii *IL1β* +3953

Neither of the allele frequencies of the single nucleotide polymorphisms in *IL1B* at +3953 bases was found to differ significantly between the two groups.

GENOTYPE	CONTROLS	CASES		ALLELES	CONTROLS	CASES
1:1 (T/T)	208 (0.72)	152 (0.73)		1 (T)	499 (0.837)	494 (0.846)
1:2 (T/C)	74 (0.25)	52 (0.25)		2 (C)	97 (0.163)	90 (0.154)
2:2 (C/C)	9 (0.03)	4 (0.02)				
Total	291	208		Total	596	584

Chi square by genotype wise analysis is χ^2 0.69, DF 2 p-value 0.7, and by allele-wise analysis is χ^2 0.26 DF 1 p value 0.61 which is not significant, Odds ratio 1.1 and heterozygosity of 0.27.

Table 6.3.3iii *IL1B*-+3953

6.3.4i *IL4* VNTR

None of the three genotypes of the polymorphic 70bp repeat was found to differ in distribution between groups.

GENOTYPE	CONTROLS	CASES
1:1 (183bp)	53 0.18)	43 (0.19)
1:2 (183/253bp)	143 (0.48)	99 (0.44)
2:2 (253bp)	103 (0.34)	83 (0.37)
Total	299	224

Chi-square by genotypes, 0.68 DF 2 p-value 0.71 NS Heterozygosity = 0.49

Table 6.3.4i *IL4* VNTR

6.3.4ii *IL9* (TG)n

Eleven alleles were observed in this study (Table 6.3.4ii). Allele 12 was not observed in this population. Distribution of allele 2 differed significantly between controls and cases (p=0.036). Allele 2 was more frequent in control (0.178) than in cases (0.13). Also the frequency of allele 5 differed significantly between controls and cases p=0.0005 being more frequent in cases (0.38) than controls (0.28). Thus, this study reports IL-9 allelic association with TB in humans. The overall χ^2 19.41, DF 10 p=0.035 and was still very significant after Bonferroni (B) adjustments (0.004).

ALLELE	CONTROLS	CASES	χ^2	p-value	Odds ratio
1	15 (0.025)	16 (0.03)	0.26	0.6	0.78
2	108 (0.18)	66 (0.13)	4.36	0.036	1.44
3	131 (0.215)	110 (0.22)	0.00	0.99	0.99
4	94 (0.155)	67 (0.13)	0.95	0.33	1.2
5	172 (0.28)	194 (0.38)	12.06	0.0005	0.64
6	56 (0.091)	30 (0.06)	3.76	0.05	1.61
7	15 (0.025)	16 (0.03)	0.26	0.6	0.78
8	3 (0.005)	2 (0.01)	0.932	0.33	1.52
9	6 (0.01)	3 (0.006)			
10	7 (0.012)	2 (0.004)			
11	1 (0.002)	0 (0)			
Total	608	506			

Chi-square, χ^2 19.41, DF 10, p-value **0.035** S Heterozygosity =0.81. Alleles 8, 9, 10 and 11 were collapsed into one group.

Table 6.3.4ii *IL9* (TG)n

6.3.4iii *IL9* T113M RFLP

Given the interesting result of the microsatellite marker above a second *IL9* polymorphism in the coding region of the gene was typed. It may be that the microsatellite alleles are in linkage disequilibrium with coding and functional variant of the gene. Unfortunately, the allele frequencies for this *IL9* RFLP did not differ significantly between the two groups of cases and controls. The p-value was 0.85 and the Chi-square was 0.038.

ALLELE	CONTROLS	CASES
1 (C)	0.84	0.84
2 (T)	0.16	0.16

Chi Square, χ^2 0.038, DF 1, p 0.85

Table 6.3.4iii *IL9* T113M

6.3.4 Haplotype frequencies for the two *IL9* markers (TGn and SNP)

Although, there was no significant difference in the haplotype frequencies between cases and controls for the *IL9* RFLP, the haplotype frequencies for the two *IL9* markers gave a significant p value of 0.03 for both microsatellite and SNP. The most common haplotype is the allele 5 of TG repeat and allele 1 of SNP. The alleles of microsatellite are on the outer side followed by alleles of the SNPs see section 6.3.2v for more details on haplotype analysis.

Haplotypes	CONTROLS	CASES
1-1	0.022	0.028
2-1	0.14	0.11
3-1	0.19	0.18
4-1	0.13	0.09
5-1	0.25	0.32
6-1	0.07	0.07
7-1	0.02	0.03
8-1	0.002	0.002
9-1	0.005	0.002
10-1	0.005	0.004
11-1	0.002	0.0
1-2	0.003	0.009
2-2	0.04	0.02
3-2	0.03	0.02
4-2	0.03	0.02
5-2	0.04	0.07
6-2	0.02	0.006
7-2	0.002	0.010
8-2	0.003	0.00
9-2	0.0014	0.00

Chi square χ2 31.8, DF 19, p value 0.03

Table 6.3.4 Haplotype frequencies for *IL9* markers

6.3.5 *LTA* and *TNFA* promoter polymorphisms

There was no significant differences in allele distribution between cases and controls for LT intron 2 polymorphism,(0.2 DF 1, P=0.64), *TNFA*–863 G to T transversion, (χ^2 0.11 DF 1, p=0.23), *TNFA* -857 C to T transition (χ^2 1.15, DF 1 p=0.523) or the *TNFA*–376 G to A transition (χ^2 0.2 DF 1 p=0.85). For the *TNFA*–308 G to A transition, allele 1 was significantly more frequent in cases 0.86 than in controls 0.81 (χ^2 3.3, DF 1, p=0.018), Odds ratio 1.44. Also, the distribution of alleles for *TNFA*–238 G to A transition differed significantly between groups. Allele 2 the rarer allele, was more frequently seen in cases (0.09) than in controls (0.05) whereas the more common allele 1 was more frequent in controls (0.95) than cases (0.91). The overall association between groups was significant (χ^2 =26.73, DF 1, p 0.016) after Bonferroni adjustments (0.025 for biallelic polymorphism). The *TNFA*-promoter region alleles were found to be in Hardy Weinberg equilibrium. The tables below shows the genotype and allele frequencies for the different *TNFA* polymorphisms.

6.3.5i *LTA*

None of the alleles differ significantly in their distribution.

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
GG	39 (0.14)	28 (0.13)		1G (Asn 26)	225 (0.39)	163 (0.37)
GA	147 (0.51)	107 (0.48)		2A (Thr 26)	349 (0.61)	277 (0.63)
AA	101 (0.35)	85 (0.39)				
Total	287	220		Total	574	440

Chi-square by genotypes, χ^2 0.64, DF 2, p-value 0.73. Chi square by allelesχ^2 0.22 DF 1 p-value 0.637
Odds ratio 0.939 Heterozygosity = 0.48.

Table 6.3.5i *LTA* by genotypes and alleles

6.3.5ii *TNFA* 863

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
C/C	216 (0.84)	239 (0.88)		G	471 (0.91)	509 (0.93)
C/A	39 (0.15)	31 (0.11)		T	45 (0.09)	37 (0.7)
A/A	3 (0.01)	3 (0.01)				
Total	258	273		Total	516	546

Chi-square by genotypes, χ^2 0.14, DF 2, p-value 0.9. Chi square by alleles, χ^2 0.11, DF 1 p value 0.23
Odds ratio 1.04, Heterozygosity = 0.14

Table 6.3.5ii *TNFA* –863 C/A SNP

6.3.5iii *TNFA* -857 C/T

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
CC	238 (0.89)	249 (0.87)		C	506 (0.94)	531 (0.93)
CT	30 (0.11)	33 (0.12)		T	30 (0.06)	37 (0.07)
TT	0 (0.0)	2 (0.07)				
Total	268	284		Total	536	568

Chi square by genotypes, χ^2 1.89, DF 2, p-value 0.39, Chi square by alleles χ^2 1.15 DF 1 p value 0.52
Odds ratio 0.75 NS Heterozygosity =0.113

Table 6.3.5iii *TNFA* –857 C/T SNP by genotype and allele

6.3.5iv *TNFA* -376 G/A SNP

Homozygotes for the mutant genotype A is rare and was not seen in the cases and only one out of two hundred and sixty eight samples was observed in the control group.

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
GG	255 (0.95)	274 (0.94)		G	522 (0.97)	562 (0.98)
GA	12 (0.05)	14 (0.06)		A	14 (0.03)	14 (0.02)
AA	1 (0.0)	0 (0)				
Total	268	288		Total	536	576

Chi square by genotypes, χ^2 0.22 DF 1, p-value 0.64 S Odds ratio 0.84, Chi square by alleles χ^2 0.22, DF 1 p-value 0.85 NS Odds ratio 0.84 NS Heterozygosity =0.05

Table 6.3.5iv *TNFA* –376 G/A SNP by genotype and allele

6.3.5v *TNFA* -308 G/A

The TNF –308 polymorphism has the highest heterozygosity in this population of the TNF variants analysed. The heterozygosity is 0.31. The frequency of allele 1 (G) is more in TB cases (p=0.018).

GENOTYPE	CONTROLS	CASES		ALLELE	CONTROLS	CASES
GG	202 (0.67)	184 (0.76)		G	489 (0.81)	417 (0.86)
GA	85 (0.28)	49 (0.20)		A	113 (0.19)	65 (0.14)
AA	14 (0.05)	8 (0.03)				
Total	301	241		Total	602	482

Chi-square, χ^2 5.57 DF 2 p-value 0.06, Chi-square, χ^2 3.87, DF 1 p-value **0.018,** Odds ratio 1.44 S Heterozygosity= 0.31.

Table 6.3.5v *TNFA* –308 G/A SNP by genotypes and alleles

6.3.5vi *TNFA* -238 G/A

The distribution of both the genotypes and alleles of this polymorphism differed significantly between cases and controls. Allele 2 (A) of this polymorphism is significantly associated with TB from this study (p=0.016).

GENOTYPE	CONTROLS	CASES	χ^2	p-value	Odds ratio
GG	200 (0.91)	179 (0.82)	52.68	0.01	4.544
GA	18 (0.08)	40 (0.18)	41.77	0.01	0.244
AA	2 (0.009)	0 (0)	6.27	0.01	0.208
Total	220	219			

Chi square by genotype, χ^2 11.51, DF 2, p-value 0.003

Table 6.3.5via *TNFA* -238 G/A SNP by genotypes

ALLELE	CONTROLS	CASES
G/G	418 (0.95)	398 (0.91)
A/A	22 (0.05)	40 (0.09)
Total	440	438

Chi square by alleles, χ^2 26.73 DF 1 p-value <**0.016** Odds ratio 1.91, Heterozygosity = 0.11.

Table 6.3.5vib *TNFA* -238 G/A SNP by alleles.

6.3.5vii Haplotype analysis of the *TNFA* region

In collaboration with Hans Ackerman and Professor D Kwiatkowski, the *TNFA* promoter SNPs by haplotye analysis was carried out. The program (PHASE) used was developed by Mathew Stephens working with Professor Peter Donnelly in the department of statistics and in Oxford. Individuals for which PCR failed were excluded. This resulted in small sample size of 154 cases and 174 controls. Fourteen haplotypes were observed in this population. The SNP order is as follows: For example, haplotype 1 which is the most common haplotype with the SNP order ACCGGG has A (adenine) nucleotide substitution for the wild type *LTA NcoI* variant, C (cytosine) for the wild type *TNFA*-863 variant, C (cytosine) for the wild type *TNFA* -857 variant, G (guanine) for the wild type *TNFA* -376 variant, G (guanine) for the wild type *TNFA* -308 variant, and lastly G (guanine) for the wild type *TNFA* -238 variant. Linkage disequilibrium occurs more in the wild type alleles for all the *TNFA* SNPs. The frequency of haplotype 4 consisting of GCCGAG is significantly different between cases and controls. This haplotype occurs more in controls than in cases. The haplotype 4 consists of the mutant LTA allele 1 (G) that in this study is allele 2, mutant *TNFA* -308 allele 2 (A), and the *TNFA* -238 wild type allele 1 (G). The linkage disequilibrium exhibited by these alleles supports our earlier association of *TNFA* –308 allele 1 (G) and allele 2 (A) of *TNFA*-238 with TB. Haplotype 9 differs from haplotype 4 at three points; containing opposite nucleotide variants for the *LTA* wild type (A) nucleotide, wild type G nucleotide variant for *TNFA* -308, and mutant variant A at *TNFA* -238. These two haplotypes were observed more in controls.

Haplotype	SNP order	Control	Cases	OR	C.I (95%)	p-value
1	ACCGGG	141(0.41)	151(0.49)			
2	GCCGGG	65(0.19)	70 (0.23)			
3	GCCAAG	2 (0.01)	5 (0.02)			
4	GCCGAG*	65 (0.19)	35 (0.11)	0.56	0.35-0.89	0.009
5	ACTGGG	21 (0.06)	20 (0.06)			
6	AACG*GG*	25 (0.07)	18 (0.06)			
7	ACCAGA	6 (0.02)	0 (0.00)			
8	ACCAGG	2 (0.01)	6 (0.02)			
9	ACCGGA*	13 (0.04)	0 (0.00)	0.00	0.00-0.36	0.0006
10	GCTGAG	1 (0.00)	1 (0.00)			
11	ACCGAG	3 (0.01)	1 (0.00)			
12	GACGAG	2 (0.01)	0 (0.00)			
13	GACGGG	1 (0.00)	1 (0.00)			
14	AATGGG	1 (0.00)	0 (0.00)			
		348	308			

The table shows haplotype frequency in both cases and controls and compares a χ^2 for these haplotypes

* Haplotypes 4 and 9 with opposite nucleotide changes are more frequent in controls.

Table 6.3.5vii Haplotype analysis of the *TNFA* region.

6.3.5viii TNFa

TNFa is a microsatellite marker in the TNF gene cluster of the MHC class III molecule. Fourteen alleles were observed in this population during this study. A trinomial distribution of alleles was observed. The alleles 2, 6 and 11 occurred more in both cases and controls and they were at equal frequency. Alleles 5, 8, 9, 12 & 14 were collapsed. Neither of the TNFa microsatellite alleles differs significantly in distribution.

ALLELES	CONTROLS	CASES
1	16(0.027)	9(0.017)
2	105(0.18)	73(0.14)
3	27(0.046)	27(0.054)
4	34(0.06)	37(0.08)
5	12(0.02)	10(0.02)
6	126(0.21)	123(0.23)
7	70(0.12)	54(0.11)
8	3(0.005)	1(0.002)
9	10(0.017)	8(0.015)
10	60(0.1)	71(0.13)
11	125(0.21)	102(0.19)
12	0(0)	7(0.001)
13	0(0)	0(0)
14	4(0.005)	6(0.011)
Total	592	528

Chi- square, χ^2 9.72 DF 8 p value 0.28 NS Heterozygosity=0.85.

Table 6.3.5viii TNFa by alleles

6.3.6i *IFNGR1* (TT) deletion

A dinucleotide deletion of thymine (ddelTT) in the *IFNGR1* detected during this study was analysed by genotype-wise and allele-wise methods. Although there was no significant difference in allele frequencies between the groups, owing to the low frequency of this polymorphism (χ^2 0.66 DF 1 p=0.42 Odds ratio 0.84), there was a significant association between controls and cases when genotypes were considered. The double deletion homozygote mutant was observed more in TB cases (0.09) than in controls (0.01) and the frequency differed significantly between groups. The frequency of heterozygote was more in controls (0.17) than cases (0.10) but this difference was not statistically significant. Overall χ^2 16.88 DF 2 p=0.0002 and was significant after Bonferroni (B) adjustments (0.016). This association may be spurious because of low frequency of the deletion but the fact the polymorphism occurs at a potential transcription factor binding (GAS) site makes it very interesting and more studies will be required to confirm its biological significance in TB.

GENOTYPE	CONTROLS	CASES	χ^2	p-value	Odds ratio
TT/TT	243 (0.82)	114 (0.81)	0.01	0.92	0.99
TT/--	51 (0.17)	14 (0.10)	3.27	0.07	0.54
--/--	4 (0.01)	12 (0.09)	112.16	0.0004	0.6.89
Total	298	140			

Chi square by genotypes, χ^2 16.88 DF 2 p-value 0.0002 S Heterozygosity=0.18

Table 6.3.6ia *IFNGR1* (TT) deletion by genotypes

ALLELE	CASES	CONTROLS
TT	242 (0.86)	537 (0.9)
--	38 (0.14)	59 (0.1)
Total	280	596

Chi square by alleles χ^2 0.66 DF 1, p-value 0.42 Odds ratio 0.84

Table 6.3.6ib *IFNGR1* (TT) deletion by alleles

6.3.7 *IFNG*

This is the only type able polymorphism in the human *IFNG* gene. None of the alleles of this microsatellite were associated with TB.

ALLELE	CONTROLS	CASES
1	49 (0.08)	44 (0.075)
2	134 (0.21)	127 (0.215)
3	277 (0.44)	261 (0.443)
4	39 (0.062)	33 (0.056)
5	62 (0.163)	63 (0.107)
6	21 (0.033)	26 (0.044)
7	35 (0.055)	28 (0.05)
8	9 (0.014)	3 (0.005)
9	4 (0.006)	3 (0.005)
Total	630	588

Chi-square,χ^2 4.44 DF 8, p-value=0.814, NS Heterozygosity=0.74.

Table 6.3.7 *IFNG* by alleles

6.3.8i *NOS2A*

None of the alleles of the polymorphism differ significantly in distribution between cases and controls. There was a binomial distribution of alleles with alleles 3 and 6 being the highest frequency in this population.

ALLELE	CONTROLS	CASES	χ^2	p-value	Odds ratio
1	47 (0.08)	50 (0.096)	9.287	0.76	0.925
2	78 (0.13)	70 (0.13)	0.179	0.67	1.085
3	109 (0.18)	78 (0.15)	0.65	0.42	1.139
4	52 (0.09)	39 (0.07)	1.59	0.21	1.31
5	77 (0.13)	68 (0.13)	1.86	0.97	0.98
6	114 (0.19)	110 (0.15)	0.015	0.9	0.974
7	49 (0.088)	52 (0.21)	0.248	0.62	0.89
8	27 (0.05)	33 (0.06)	1.16	0.28	0.74
9	14 (0.024)	8 (0.015)	8.94-04	0.976	1.14
10	4 (0.006)	5 (0.009)	1.55	0.218	0.38
11	4 (0.006)	0 (0)	3.06	0.08	0.00
12	0 (0)	0 (0)			
13	0 (0)	1 (0.001)	1.58	0.21	0.00
14	11 (0.02)	8 (0.015)	2.67	0.1	0.4
15	4 (0.006)	0 (0)	0.79	0.37	0.00
Total	590	522			

Chi-square by alleles, χ^2 8.53 DF 8 p-value 0.384 NS.Heterozygosity=0.865. Alleles 9, 10, 11, 12, 13, 14, 15 were collapsed into one group.

Table. 6.3.8i *NOS2A* by alleles

6.3.8ii D17S250

None of the alleles of the marker D17S250 differed significantly in distribution. Allele 12 had the highest frequency in this population. Allele 5 had the second highest frequency.

ALLELE	CONTROLS	CASES	χ^2	p-value	Odds ratio
1	4 (0.0065)	2 (0.0038)	2.76-^{02}	0.0867	1.22
2	40 (0.065)	35 (0.067)	2.43-^{03}	0.96	1.04
3	98 (0.16)	62 (0.12)	2.23	0.135	1.276
4	38 (0.06)	33 (0.063)	0.53	0.465	0.83
5	103 (0.17)	89 (0.17)	2.159	0.88	1.032
6	86 (0.14)	63 (0.12)	2.45	0.1173	1.307
7	34 (0.06)	31 (0.056)	0.063	0.801	0.918
8	45 (0.07)	28 (0.05)	4.30	0.03	1.632
9	6 (0.0098)	23 (0.04)	9.458	0.0002	0.254
10	5 (0.008)	5 (0.0095)	5.26	0.02	3.47
11	5 (0.008)	7 (0.013)	1.231	0.267	0.404
12	134 (0.22)	135 (0.26)	2.53	0.11	0.81
13	0 (0)	0 (0)			
14	0 (0)	1 (0.019)	9.822	0.001	0.00
15	4 (0.0065)	0 (0)	5.29	0.021	0.00
16	3 (0.0049)	0 (0.003)			
17	4 (0.0065)	11 (0.021)	2.39	0.12	0.37
18	0 (0)	1 (0.0019)	0.322	0.57	0.00
19	1 (0.0016)	0 (0.0)			
	610	526			

Chi-square, χ^2 6.87 DF 8 p-value 0.55 Heterozygosity =0.86. Alleles 1,10,11,15, 16, 17, 19 were collapsed into one group.

Table 6.3.8ii D17S250 by alleles

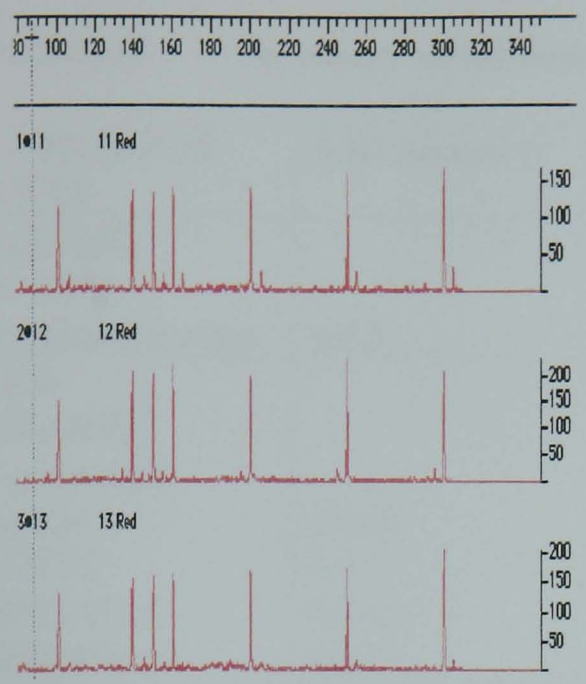


FIG.6.2 Analysis of the internal size standard using genotyper 2.0. The corresponding size of an allele is determined relative to the ROX internal size marker and is indicated using the scale above.

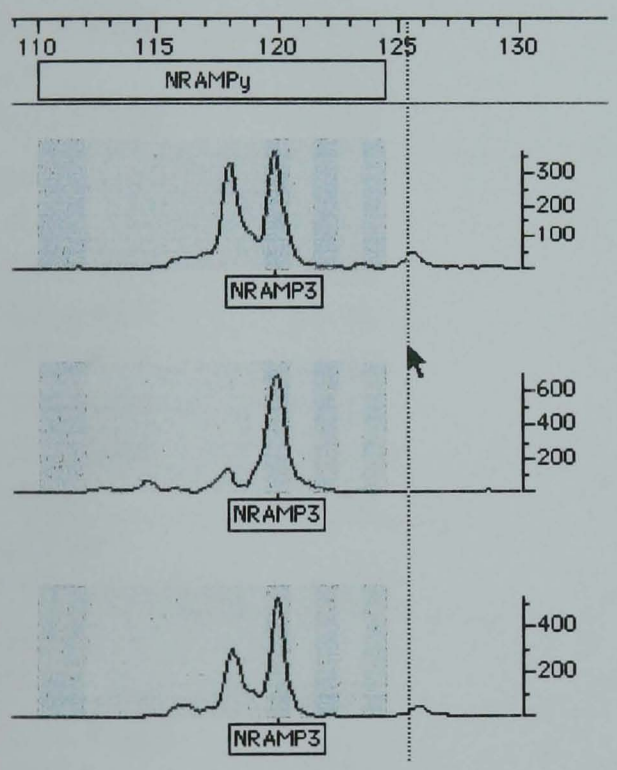


FIG. 6.3 Analysis of the NRAMP1 microsatellite using Genotyper 2.0. The relative intensity of fluorochrome is indicated on the right hand side of the graph.The dark gray bars indicate the positions of the alleles. This graph show genotype for three individuals. All three examples are homozgotes for the common allele3.

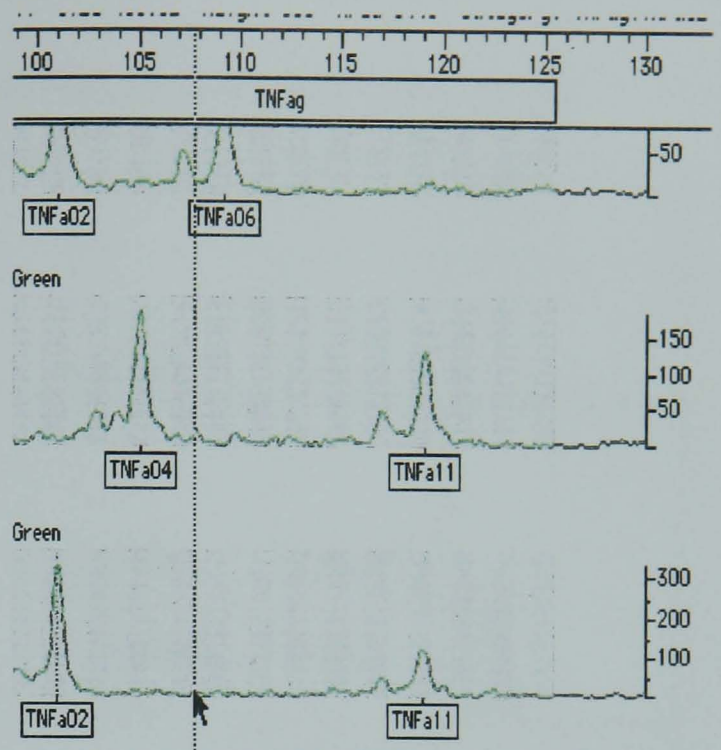


FIG.6.4 Analysis of the TNFa microsatellite using the Genotyper 2.0. The TNFa is a dinucleotide repeat and is more polymorphic than the NRAMP1 microsatelite. Individuals were more likely to be heterozygous.

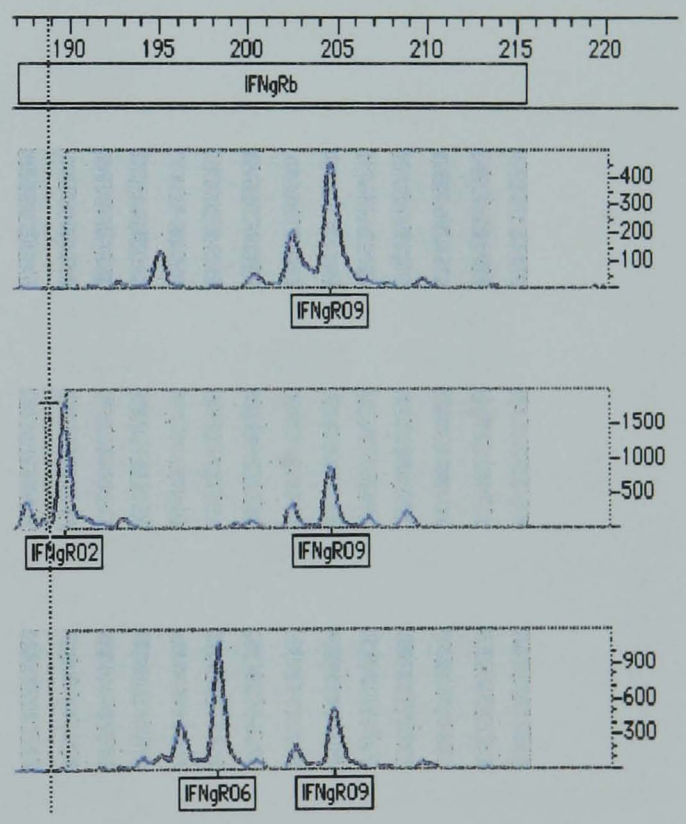


FIG. 6.5 Analysis of the IFNGR1 microsatellite using Genotyper 2.0. The most common allele 9 is usually seen in combination with other alleles.

Table 6.4 Summary of influence of macrophage candidate genes on TB

CANDIDATE GENE	Chi square χ^2	DF	p-value	B	OR
1q31-q32					
IL-10 5' proximal repeat	15.2	10	0.125		
2q35					
NRAMP1					
5'promoter repeat	10.36	3	0.016	0.0125	
274C/T	1.1	1	0.3		1.1
469+14G/C	15.93	1	0.00006	0.025	2.5
1465-85G/A	0.03	1	0.92		1.02
2q14					
IL1RN	5.05	3	0.16		
IL1B-511	9.33	1	0.002	0.025	1.48
IL1B+3953	0.26	1	0.61		1.1
5q23-q-32					
IL4 VNTR	0.68	2	0.71		
IL9	19.4	10	0.03	0.004	
IL9T113M	0.04	1	0.85		
6p23					
LTA	0.44	1	0.53		0.91
TNFA-863	0.04	1	0.23		1.04
TNFA-857	1.41	1	0.52		1.04
TNFA-376	0.22	1	0.85		1.11
TNFA-308	4.22	1	0.018	0.025	0.67
TNFA-238	27.73	1	0.016	0.025	1.91
TNFa	9.72	8	0.28		
6q23-24					
IFNGR1 TT/del	16.88	2	0.0002	0.016	
12q24.1					
IFNG	4.44	8	0.814		
17q11.2-q12					
NOS2A	8.53	8	0.38		
D17S250	6.87	8	0.55		

The chi-square is represented with the symbol, χ^2 DF represents the degrees of freedom, the conditional probability is represented by p-value and B represents the Bonferroni adjustments for multiple allele analysis correction of statistical significance.

The summary of result is presented for eight chromosome regions and 22 candidate genes. After correction for multiple allele analysis, two genes with allele frequency initially associated with TB were no longer significant after Bonferroni adjustments. These are the IL-9 microsatellite repeat and the *NRAMP1* CA Z DNA polymorphism. Other genes that are still associated with TB are *NRAMP1* 469+14G/C intron 4, *IL1B* -511, *IFNGR1* delTT, and two *TNFA* promoter polymorphisms at TNFA-308 and *TNFA*-238. Opposing alleles were shown to be associated with TB.

6.4 Discussion

Twenty-two macrophage candidate genes were genotyped at eight chromosome regions. *IL10* on chromosome 1q31-q32, *NRAMP1* on 2q35, IL-1 on 2q14, Th2 cytokines *IL4* and *IL9*, *LTA* and *TNFA* on the short arm of chromosome 6 within the MHC class III region, *IFNG* on chromosome 12q24.1 and *NOS2A* and the small inducible chemokines on chromosome 17q11.2-q12.

This study demonstrates association between TB and two *NRAMP1* polymorphisms studied. Of the four polymorphisms analysed in this study, two had been reported to be associated with TB. The promoter Z DNA repeats and INT 4 (*NRAMP1* 469+14 G/C in this study) polymorphisms were reported to be significantly associated with TB in this population (Bellamy *et al.,* 1998b). The results obtained from this study are similar to that of the Bellamy study. Although, the promoter polymorphism showed statistically significant difference initially using χ^2 test analysis, after Bonferroni adjustments it just statistically significant. Similarly, studies in mice also failed to show any effect of a protective role for the *Nramp1* gene after *M. tuberculosis* infection (North *et al.,* 1999, Medina and North, 1996). The *NRAMP1* intron 4 alleles showed significant difference in distribution between the two groups. Supporting earlier reports of allelic association with TB.

It may be that polymorphisms within this interval are associated with TB and it may be important to find out if there is any linkage disequilibrium between alleles of these *NRAMP1* polymorphisms and other neighbouring markers. IL-8 has been suggested as possible candidate gene for TB susceptibility (Friedland *et al.,* 1995). Demonstrations of elevated levels of IL-8 mRNA and protein in supernatants of macrophages and in lavage fluid obtained from patients with pulmonary tuberculosis

may suggest a protective role for IL-8 in TB. Indeed genetic studies in a Brazilian population have weakly linked *IL8RB* with susceptibility to TB (Shaw *et al.,* 1997). This gene is tightly linked to *NRAMP1* (*IL8RB* and *NRAMP1* are encoded on the same chromosome and are 100 kb apart) and recently, four polymorphisms within the *NRAMP1* gene have been associated with TB (Bellamy *et al.,* 1998b). Taken together these may indicate important roles for both *IL8RB* and *NRAMP1* in TB.

Of significant importance is the finding that *NRAMP1* (469+14 G/C) polymorphism is not only significantly associated with TB, it is also shown to be influencing LAM induced IL-10 responses (Table 5.4). Soo *et al.,* (1998) using congenic mice investigated the influence of host genetics on the ability to induce protective immune response to recombinant salmonella vaccines. They showed that mice carrying the wild-type *Nramp1* allele mounted a predominant T helper-1 response to vaccination whereas mice carrying the mutant *Nramp1* mounted a T-helper-2 response and showed exacerbated lesion growth upon challenge (Soo et. al., 1998).

There is evidence from this study that carriage of one copy of the *ILRN* allele 2 may confer protection against TB. The observed frequency of allele 2 is low in The Gambian population (0.04) from this study (Table 6.3.3i) and (0.025) from a previous study (Bellamy *et al.,* 1998d). In Caucasians, it occurs at a much higher frequency (0.24) (Clay *et al.,* 1994, Mansfield *et al.,* 1994) where it has been associated with numerous autoimmune diseases.

In contrast to an earlier report by Bellamy *et al.,* (1998d) this study failed to demonstrate any association between with *IL1RN* allele 2 and TB. Much greater sample size will be required to determine whether homozygotes for allele 2 have altered susceptibility to tuberculosis in this population.

However, *IL1B* result from this study is supported by an earlier study by Wilkinson *et al.,* (1999) which demonstrated an association between IL-1β and TB. The Wilkinson study demonstrated significant association between an allele of *IL1B* +3953 and TB in a panel of migrant Indians, this study however, demonstrates significant association between allele1 of the *IL1B* -511 polymorphism and TB in The Gambian population (Table 6.3.3ii). Although, these studies were carried out in two different populations with different genetic background, the fact that the same gene but different mutants has been associated with TB may suggest important role for IL-1β in susceptibility to TB.

This study also identified an association between TB and allele 5 of the *IL9* microsatellite (Table 6.3.4iii). Linkage disequilibrium data in humans suggests *IL9* may be associated with atopy (Doull *et al.,* 1996). In mice, particular alleles of *IL9* have been associated with bronchial hyper-responsiveness and atopy (Nicolaides *et al.,* 1997). A study in 1996 reported an association between allele 118 of *IL9* and atopy in humans (Doull *et al.,* 1996). Inverse relation between atopy and DTH has been reported in Japanese school children (Shirakawa *et al.,* 1997) leading to the hypothesis that opposing factors may be responsible for DTH in TB and atopy. This study now reports *IL9* allelic association with TB in the Gambian population. Another *IL9* polymorphism (T113M) typed was not found to be associated with TB. Haplotype analysis gave a significant p value of 0.03 for both the (TG)n microsatellite and the T113M SNP.

Despite conflicting reports of *TNFA* allelic association with several diseases, this study demonstrates significant association between alleles 1 *TNFA*-308 (G) and allele

2 *TNFA* –238 (A) and TB. Earlier studies of *TNFA*-308 polymorphism have shown that for the *TNFA* –308 polymorphism, the *TNFA*-308A allele is associated with increased TNF-α transcription rates (Wilson *et al.,* 1997, Kroeger *et al.,* 1997). Association studies carried out in The Gambia found that individuals homozygous for the *TNFA* -308A allele carried a seven fold higher risk of death or severe neurological sequelae from cerebral malaria, independently of the presence of predisposing MHC class I and class II alleles (McGuire *et al.,* 1994). The pathology of cerebral malaria is associated with increased levels of circulating TNF. Also, using the same set of samples, McGuire *et al.,* (1999) reports an association between low plasma TNF levels in severely anaemic children with *Plasmodium falciparum* infection and *TNFA* -238A allele in Gambian children. This indicates that different forms of a disease may be associated with different alleles of the same gene and highlights the biological importance of single nucleotide polymorphisms and diseases. Similarly, this study also demonstrates associations between TB and two *TNFA* polymorphisms. The *TNFA* -308A (Table 6.3.5v, p = 0.18) and the *TNFA* -238G (Table 6.3.5vi, p=0.016) were associated with TB. Thus, this study has associated different polymorphisms of the same gene with TB. If *TNFA* -308A is associated with higher TNF levels (Wilson *et al.,*1997, Kroeger *et al.,* 1997) and *TNFA* -238A is associated with lower TNF levels (McGuire *et al.,* 1999), this report may strengthen the hypothesis that individuals with TB have sub optimal macrophage function and therefore reduced capacity to produce TNF. However, TNF levels were not shown to be lower in recovered cases than in controls (Table 3.5) nor was the *TNFA*-gene shown to be influencing measured TNF levels using the whole blood assay (Table 5.3.4iv).

Homozygotes for the *IFNGR1* dinucleotide deletion polymorphism had genotype frequency of 0.01 in controls and a frequency of 0.05 in TB cases (Table 6.3.6i, p=0.015). Indication of a role for the *IFNGR1* in susceptibility to intracellular diseases comes from independent studies in 1996 firstly, of Maltese children who suffered from atypical mycobacterial infection in England by Newport *et al.,* (1996) and then of a Tunisian child by Jouangay *et al.,* (1996) in France. These studies demonstrated a lack of IFNγR1 protein on cell surface of children suffering form atypical mycobacterial infections due to mutations in coding regions of the gene. This study demonstrates an association between the newly detected double deletion polymorphism (Table 4.4, FIG. 4.13) and TB in an adult African population.

None of the markers on chromosome 17, where *NOS2A* and *SCYA* (such as *RANTES*) are located were found to be associated with TB in this study. It is not surprising that this study was unable to demonstrate allelic association between alleles of *NOS2A* polymorphisms and TB. The role of NO in macrophage killing of *M. tuberculosis* though well established in mice is still unclear in humans. In contrast, the frequency of a 11 repeat of *NOS2A* CCTTT promoter variant was significantly more in fatal cases of cerebral malaria when compared with other subtypes of malaria in The Gambian patients (Burgner *et al.,* 1998). The D17S250 marker is reported to be close to the small inducible chemokine genes. None of the alleles of this marker demonstrated statistically significant difference in frequency between controls and TB cases in this study. However, this result is surprising because a locus on human chromosome 17 is linked to TB in a family based genomic screening (Lim *et al.,* 2000).

The promoter repeat polymorphism of *IL10* and the intron one repeat of *IFNG* were not found to be associated with TB in this population and this is in agreement with an earlier study carried out in this population (Bellamy, 1998a)

In conclusion allelic associations were observed between alleles at the following candidate gene loci and TB in this study: allele 1 of *TNFA*-308, allele2 of *TNFA*-238, allele 2 of DDelTT of *IFNGR1,* allele 1 of IL-1β –511, *NRAMP1* (469+ 14 G/C), allele 5 of *IL9* intron 4 (TG)n marker.

CHAPTER 7

DISCUSSION

7.1 Introduction

Defects in specific components of the immune system have provided many clues to the complex immunological mechanisms underlying resistance or susceptibility to infection. A beneficial response by host to mycobacterial infection involving both the innate and acquired arms of the immune system is an important determinant of disease resistance. This response is subject to genetic regulation. The innate immune system represents the first line of defense against invading organisms. Studies in animals and humans suggest that macrophage activation is essential to killing of mycobacteria and containment of mycobacterial infection. The macrophage activation process or pathway involves complex networking of several molecules. Due to the complexity of this process, the quantitative phenotype analysis is a useful tool for teasing out underlying quantitative traits, which can then be analysed separately. Quantitative phenotype analysis has been used to identify genetic loci regulating complex diseases including asthma (Daniels, 1996). The identification of the real disease gene(s) among candidates relies upon the characterisation of specific defects in affected individuals. Essentially, this study was set up to search for evidences of genetic markers correlating with cytokine secretion as a marker of macrophage activation and thereafter determine whether these genes influence TB. This study is the first to employ this method in identifying TB susceptibility genes in humans.

7.2 Monocyte function in The Gambian population.

Whole blood of persons who had TB and healthy blood donors were stimulated with bacterial cell wall components. Pro inflammatory (TNF and IL-1β) and anti-inflammatory (IL-10) cytokines, induced by macrophage stimulating agents were determined as quantitative traits. Levels of IL-10 induced in response to LPS differed between recovered TB cases and controls with IL-10 levels being higher in cases ($p=0.02$). This study demonstrates that macrophage activation is a heterogeneous process with much inter-

individual variation. This implies that regulation of this process is dependent in part on the individual's genetic constitution. Macrophage activation pathways involve a complex networking of several molecules all acting together with many possible points of control of synthesis and function. Published functional polymorphisms at macrophage candidate gene loci were studied for any influence on quantitative traits in The Gambian population. It was later determined whether those found to be regulating microbial induced responses also influenced TB susceptibility.

Positive correlations for cytokine production were observed for LAM and LPS (both microbial wall components known to activate macrophage/monocytes). This suggests a common pathway of action and implies that they activate macrophage through the same cell surface receptors see section 3.3.3 or pathway. The same genes or pathway may be involved in initiating the macrophage activation pathway. There was significant correlation between TNF and IL-1β production induced by either of the stimulants. This suggests again that dependent pathway or genes may be essential for the release of these cytokines. This indicates that the intracellular processes required for the production of these molecules though different at certain points may have overlapping function and therefore are important determinants of macrophage containment or killing of mycobacteria.

The CD14 molecule and TLRs are germ-line-encoded receptors of the innate immune system referred to as pattern recognition receptors (PRR). They are both present on macrophages and there are described polymorphisms for both genes but they were not typed during this study. A mutation designated Asp299Gly interrupts TLR4- mediated LPS signaling (Arbour *et al.*, 2000) There are reported polymorphisms for the CD 14 molecule but one described C→T polymorphism at position –260 bases appears to be absent in the panel of Gambians studied (Hubacek *et al.*, 1999).

Interferon-γ differentially modulates LPS induced expression of transcription factor activator protein –1 (AP-1) and NF-Kβ at the mRNA and protein levels in human monocytes. Priming of human monocytes with IFN-γ results in the down regulation of c-*fos* and c-*jun* mRNA in response to stimulation with LPS whereas the inflammatory molecule NF-Kβ was up regulated. Also DNA binding capacity of AP-1 is reduced. However an increased NF-kβ expression was noticed in IFN-γ pretreated monocytes (de Wit *et al.,* 1996). This might explain the enhancing effect on TNF levels in response to LPS stimulation by IFN-γ priming of monocytes.

7.3 Novel polymorphisms

IFNγ acting via its receptor is important in macrophage activation but there was a lack of described polymorphisms and new polymorphisms had to be detected. Ten new polymorphisms were detected in all. Five novel transition base changes were detected in the promoter region of *IFNG*. For the *IFNGR1*, a deletion polymorphism and four base changes were detected. Two polymorphisms detected in the promoter of *IFNGR1* could be functional. One, a double deletion at position –470/-471 bases, occurs at a transcription binding site (GAS) and the other a C→T transition at position -56 bases is about four bases away from a putative NF-Kβ binding site. Except for one intron six repeat and a Taq1 polymorphism in *IFNGR1* gene, other reported variants are rare and effectively lethal inactivating mutants. Also, the *IFNG* gene is not known to be extensively polymorphic. There was only one reported intron one repeat, only recently a mutant in the promoter of the gene at nucleotide position –333 of the initiation codon was reported. Specific regions of the promoter of these two genes were sequenced in thirty-six Gambian individuals. The finding of base changes in and around a transcription factor binding site implies that such variation may affect the way a transcription factor binds to that site and

subsequently the of rate transcription of the gene. Detection assays for some of these mutations were developed for use in screening the larger population.

7.4 Genetic regulation of cytokine responses

It was determined whether particular alleles at candidate gene loci are associated with measured levels of pro-inflammatory and anti-inflammatory cytokine production in response to LPS and LAM. Evidence for genetic regulation of immune response induced by LPS and LAM were observed at three candidate genes. Individuals who are homozygous for the *LTA NcoI* polymorphism allele 2 (*TNFB*1* of previous studies) produced lower levels of TNF and IL-1β in response to both LPS and LAM and did not upregulate their responses to IFN-γ as much as those carrying the other genotypes. Messer *et al.,* (1991) used PBL but stimulated with PHA and demonstrated lower *LTA* levels in *TNFB*1* homozygotes (Messer *et al.,* 1991). Individuals who are homozygous for this allele in the Messer study did have significantly different TNF levels. Corroborating our finding is the study of Stuber *et al.,* (1996, 1999) that showed higher circulating concentrations of TNF and higher mortality for septic patients with the *TNF-B*2* (allele 1 in this study) homozygous allele.

In addition, based on measured cytokine levels, homozygotes for the *TNFA*-308 allele 2 were shown to produce lower TNF and IL-1β levels when compared to other genotypes. There seems to be a trend in cytokine production with those homozygous for allele 1 producing higher cytokine levels than the heterozygous 1/2. These homozygous for allele 2 secreted the least amount of cytokines. However, upon statistical analysis of the mean cytokine levels among genotypes, no significant difference was found. In support of this is the sepsis study of Stuber et al, 1996 and that of Brinkman *et al.,* 1996. The Stuber study used allele-specific reverse transcriptase (RT)-PCR on donor blood samples and the Brinkman study used PMA inducible TNF promoter activity in human T and B cell lines.

These studies were unable to find evidence for the causal effect of the variation within *TNFA* -308 and disease. Other studies have demonstrated higher TNF secretion by homozygotes for the *TNFA*-308 allele 2 (Bouma *et al.,* 1996, Kroeger *et al.,* 1997). Of particular interest is the *NRAMP1* INT4 polymorphism that was shown to influence IL-10 secretion. Individuals who are heterozygous for the *NRAMP1* INT4 polymorphism produced lower levels of IL-10 upon LAM stimulation of whole blood when compared with the homozygote for allele 1. A functional study of *NRAMP1* CA repeat using a reporter gene construct demonstrated increased ability of the allele 3 to driving gene expression upon stimulation with LPS. Allele 2 was found to have opposite effect by driving reduced gene expression. Allele 3 is associated with rheumatoid arthritis and allele 2 with infectious diseases. The allele 2 of *NRAMP1* CA repeat is reported to be in linkage disequilibrium with allele 2 of another *NRAMP1* variant, the *NRAMP1* INT4 SNP in intron 4 (Bellamy *et al.,* 1998b). The homozygote 2 genotype was not observed in the panel of samples studied. This study used the whole blood model and did not demonstrate any influence of the alleles of *NRAMP1* CA repeat and cytokine production. Inefficient *NRAMP1* protein could affect processes within the macrophage that are essential to macrophage activation and therefore control of infection.

Anti-inflammatory *IL1RN* was shown to influence IL-10 secretion upon LAM and LPS stimulation. Individuals who are carriers or homozygous for the allele 2 of *IL1RN* VNTR were shown to have increased IL-1RA expression in monocyte cultures (Danis *et al.,* 1995). Allele 2 is a two 86bp repeat containing IFN silencer A and IFN silencer B transcription factor binding sites. Increased levels have been associated with several autoimmune diseases. Wilkinson *et al.,* (1999) demonstrated that IL-10 increased *IL1RN* expression in carriers of allele 2. This study demonstrates higher levels of IL-10 in individuals who are carriers of allele 2 upon LAM and LPS stimulation. IL-10 is an anti-

inflammatory cytokine and IL-1β and TNF may suppress the production of IL-10. IL-1RA competes with IL-1β for occupancy of the IL1R. Increased levels of IL-1RA would antagonise the effect of IL-1β. IL-1RA acts as an anti-inflammatory molecule by not transducing signals via this receptor whereas IL-1β is pro-inflammatory and transduces signals via its receptor. It could be that IL-1RA, by blocking the binding of IL-1β to its receptor, enhances the pathway for the generation of IL-10. Increased levels of IL-10 may be indicative of high IL-1RA levels in carriers of the allele 2. Unfortunately, the frequency of this allele was found to be too low in this population to allow for significant statistical association.

Contrasting observations by several workers in the ability of alleles of particular genes to regulate cytokine secretion have been explained in literature by the types and concentrations of stimulants used, therefore this study employed the use of the whole blood model. For the purpose of determining genetic variability it has several advantages over other methods and these have been described in chapter 3 section 1.

Most reports on gene expression have employed in-vitro assays that try to but do not exactly mimic in-vivo conditions. The reporter gene assay measures the initial process of protein synthesis and tells us whether an allele has biological function by how well it drives gene expression by relative intensity of signal from the reporter gene. The whole blood assay measures the end point of protein synthesis and takes into account all relevant checkpoints involved in protein synthesis. In addition assaying a large number of individuals may indicate truly functional genetic influence on TB or quantitative trait measurement of cytokines.

7.5 Genetic susceptibility to TB

Suprisingly, for this study, the *LTA* gene was not found to be associated with TB in this population. Rather it is the *TNFA*-238 allele 2 and *TNFA* -308 allele 1 that were shown to

be significantly associated with TB before and after applying the Bonferoni test. It could be that for TB *per se* this *LTA* polymorphism might not be relevant but might be important for macrophage function and other macrophage associated diseases such as rheumatoid arthritis where most of the genes that have been shown to be associated with susceptibility TB are associated with resistance to autoimmune disease. Also, there is a report by Lucas *et al.,* (1999) suggesting a role for *LTA* beta-receptor in host defense against *Mycobacterium bovis* BCG infection. It might be that some other components of the LT pathway are important in TB. Other results of interest within the *TNFA* loci are the positive association of particular *TNFA*-variants with TB; yet these variants were not shown to be influencing measured cytokine levels. Allele 1(G) of the *TNFA* -308 and allele 2 (A) of *TNFA* -238 variants were found to be associated with TB in this population. The result of the *TNFA* -308 from this study contrast reports from previous studies of infectious diseases. The allele 2 of *TNFA* -308 variant is associated clinically with lepromatous leprosy (Roy et al, 1997), cerebral malaria (McGuire *et al.,* 1994), scarring trachoma (Conway *et al.,* 1997) and mucocuteanous leishmaniasis (Cabrera *et al.,* 1995). However, initial analysis of a Brazilian leprosy study suggests evidence for an association with allele 1(G) of the *TNFA* -308 (Blackwell personal communication). It could be that for mycobacterial diseases TNF protects from disease but exacerbates disease in cerebral malaria, scarring trachoma and mucocuteanous leishmaniasis. However, the frequency of homozygotes *TNFA* -308 allele 2 in this population was very low to allow for significance testing. Larger sample size will be required to confirm this finding.

Interleukin 1β is shown to be associated with TB in this study. IL-1β is a proinflammatory cytokine with similar immunomodulatory functions as TNF. The frequency of promoter polymorphism *IL1B* -511 allele 1 (T) was more in TB cases than in controls. It is difficult

to tell which of the *IL1B* allele is the mutant or wild type variant because both alleles were observed at equal frequency in both cases and controls. A role for IL-1β is suggested from this study as it corroborates the Wilkinson study that associates an allele of IL-1β +3953 with TB in-migrant Indian population (Wilkinson *et al.*, 1999).

Another gene found to be associated with TB is the Th2 cytokine IL-9. Alleles of the intron 4 repeat were found to be significantly associated with disease in this population. Epidemiological studies of migrants from areas of high helminthic infestations show that such people are prone to development of TB (Bentwich *et al.*, 1999). Helminthic infestation triggers the production of IgE, and activates the immune system towards a Th2 cytokine profile and perhaps down regulating the Th1 that is beneficial for containment of TB. A recent study also shows that BCG given early in infancy may prevent the development of atopy in African children (Aaby *et al.*, 2000). High serum IgE levels are synonymous with the immediate type hypersensitivity characteristic of atopic disorders. Serum IgE levels in atopic patients have been associated with a particular allele "118" of an IL-9 marker (Doull *et al.*, 1996). Perhaps, individuals mounting predominant Th2 type immune responses favouring IgE production are more susceptible to mycobacterial infections. The genetic basis for this was investigated in our Gambian TB cases. This study shows that allele 5 of *IL9* marker was significantly associated with TB. Other published polymorphisms in the *IL9* gene were typed and serum IgE levels in TB cases are being analysed. The *IL9* (C) T113M (T) polymorphism allele 1 with variant C and allele 2 with variant T was not found to be associated with TB. Another polymorphism in the *IL9* appears to be absent in the Gambian samples typed (Holloway, personal communication). Since the *IL9* repeat shown to be associated with TB is an intronic polymorphism little conclusions can be drawn from this finding.

The interpretations of marked differences in genotype frequency in a case control association study could be either of the following: -a) that the polymorphism predisposes to disease, b) the polymorphism is in linkage disequilibrium with a disease susceptibility allele either within the same gene or a neighbouring one or c) there is a confounding factor such as sex or poor ethnic matching between cases and controls studied. Options a) and b) may represent true positive results and c) false positive. In Gambia, more men donate blood. For this study, blood donors served as healthy controls and this was presumed to be a major bias of sampling moreso as a region within the chromosome Xq showed evidence of co-segregation with TB (LOD 1.77) from an earlier study in The Gambia (Bellamy 1998a, 2000). To correct for this bias due to sex, all female cases were excluded from the analysis. Speculations are that Nramp1 functions as an ion transporter regulating and regulated by cellular metal iron levels (Searle and Blackwell, 1999).

Evidence for a link between iron metabolism and Nramp1 function in innate resistance against mycobacteria comes from work done by Gomes and Appelberg (1998). They showed that the amount of available iron correlated with Nramp1 function. It could be that Nramp1 functions by transporting iron out of the parasite harboring phagosome. In humans, there are speculations that females are more resistant to TB due to low iron stores and that men, especially mine workers, are more susceptible to TB due to availability of iron (Bellamy, 1999).

7.6 Summary

This study has employed two strategies to understand genetic regulation of immune responses to TB and macrophage activation in humans. The approach was to use functional immunological and genetic methods to understand susceptibility or resistance to TB in humans. It was proposed that people with TB might have innate defect in their ability to upregulate macrophage for effective containment of the bacilli. Genotypic

analysis of polymorphisms at candidate gene loci was therefore carried out in relation to phenotypic analysis of disease trait and quantitative trait measurements in healthy Gambian individuals to determine whether polymorphisms at these loci influence either of these phenotypes.

For the quantitative phenotype analysis, cellular studies of monocyte function were carried out using a whole blood model. Whole blood was stimulated with either manLAM or LPS in the presence or absence of rIFN-γ. Initially, whole blood conditions were optimised for The Gambian population and cytokine measurements were compared between thirty eight TB cases and matched controls. There was no difference in mean TNF and IL-1β cytokine levels between the two groups (recovered TB cases and controls). However, significant difference was observed for LPS induced IL-10 production between recovered cases and controls ($p=0.02$). This implies an important role for IL-10 in pathology of TB. A recent study of anergic TB patients demonstrated the presence of IL-10 before and after PPD stimulation but demonstrated IL-10 production only after PPD stimulation in non-anergic TB patient (Boussiotis *et al.*, 2000). It may be that PPD anergic individuals who have inherent defect in the pathways of generation of IL-10 will have reactivation TB and treatment failures.

There were many interindividual variations in cytokine profile. This suggests that individuals differ in their ability to upregulate macrophage judged by cytokine production in response to stimulants. Samples size was increased to further investigate if this interindividual variation was genetically regulated. Three hundred and twelve healthy blood bank donors were included. Whole blood assays were carried out in this group. Some comparisons of interest were revealed: a positive correlation between LPS and manLAM induced IL-1β and TNF responses, also IL-1β and TNF responses correlated for either LPS or man LAM stimulus. It can be inferred that same genes or pathways might

regulate LPS and manLAM signals and these pathways may be similar for IL-1β and TNF. Also, this study shows that priming of whole blood with rIFN-γ enhanced the production of TNF and to a lesser extent IL-1β but rIFN-γ rather suppressed the release of IL-10.

Because of the much inter-individual variation observed polymorphisms at several macrophage candidate genes were screened to determine whether macrophage candidate genes regulate microbial induced responses and whether alleles of macrophage candidate gene influence TB. Polymorphisms in genes with known biologic function as being involved in macrophage activation were selected and genotyped in both TB and unrelated blood bank controls. In all, twenty-two macrophage candidate genes were screened. The two groups were matched for ethnicity as much as possible.

To determine if alleles of these genes regulate microbial induced responses, quantitative phenotype analysis was done. *LTA* alleles were shown to significantly influence manLAM and LPS induced pro-inflammatory cytokines TNF and IL-1β secretion ($P<0.05$), whereas, alleles of *NRAMP1* (469+14) and *IL1RN* were shown to influence manLAM induced anti inflammatory cytokine IL-10 secretion ($p=0.02$).

To determine if alleles of these genes influence TB, a case control association study was also carried out in three hundred and twelve blood bank controls and two hundred and seventy-six active TB cases. All female cases were excluded from analysis. This study has observed significant association between the following candidate genes and TB. Allele 1(G) of *TNFA* –308, allele 2 (A) of *TNFA*–238, allele 1 (T) of *IL1B*, allele 2 (C) *NRAMP1* (469+14) other markers associated with TB include allele 5 of *IL9* and 2 of *NRAMP1* (CA) Z DNA polymorphism.

Ten novel polymorphisms at specific promoter regions of two candidate gene loci were identified. For some of these new polymorphisms detection assays have been developed

while others still need to be optimised. One of these polymorphisms is expected to abolish a transcription factor binding site (GAS) at which the transcription factor GAF binds to initiate transcription of *IFNGR1*. Homozygous condition of this same polymorphism was also shown to be associated with TB ($p<0.015$). Another polymorphism at –56 of the initiation codon; a C→T transition was found to be four nucleotides away from a putative NF-Kβ binding site.

This study has contributed to the understanding of the susceptibility to tuberculosis. Furthermore, the genetic regulation of macrophage activation in humans is poorly understood. This study has made an original contribution to both of these fields. Conclusions that can be inferred from this study are that ten new polymorphisms have been detected, five within the promoter of *IFNG* and an additional five within the promoter of *IFNGR1*. It appears that similar genes or activation pathways may regulate LPS and ManLAM signals in view of positive correlation between induced TNF and IL-1β response. Alleles of different variants of the same gene (*TNFA* -308 and *TNFA* –238 polymorphism) were associated with TB in the Gambian population. Significant low levels of proinflammatory cytokine released by *LTA* allele 1 homozygotes upon stimulation of wholeblood with LPS or LAM may suggest that alleles of the *LTA* regulate microbial induced macrophage response of TNF and IL-1β production.

7.7 Future work

This study has concentrated on genetic regulation of susceptibility to TB by macrophage candidate genes. However, efficient immune responses to TB require both arms of the immune system. This work could be carried on further by looking at genes involved in other areas of innate immunity and the acquired immune responses to TB. Results form both this and these ones mentioned above will give a more comprehensive data on genes that may regulate susceptibility / resistance to TB.

Future studies may be to quantify cell surface expression of some known innate receptors which may be used by both LPS and ManLAM i.e. the CD14 molecule and TLRs 2 and 4 and to see if there is a correlation between cytokine release and cell surface receptor expression. Identification of novel polymorphisms at these loci could be used to study whether variations at these loci influence cytokine production. Another approach may be to study cytokine production in the absence and presence of these cell surface receptors to determine if these receptors are utilised by stimulants and therefore needed for cytokine production. Kinetics of mRNA expressions of these cytokines after stimulation may be studied. As this study has demonstrated significant correlation in TNF and IL-1β responses, polymorphisms in genes involved in intracellular trafficking of signals maybe studied as this may provide clues to mechanisms involved in regulating these responses. Population screens of newly detected *IFNG* and *IFNGR1* polymorphisms could be carried out to establish their frequency in The Gambian population and several African populations. Polymorphisms that appear to be influencing microbial induced responses and TB may then be analysed in a second sample set to confirm the associations observed in this study. Other sub Saharan African populations in West and East African (Nigeria and Kenya) where the ethnic admixture is minimal may be investigated with the aim of carrying out studies which may be designed to include twin, family, case control association or affected sibs studies. In the western part of Nigeria, for example, among the Yoruba's, the twinning rates are higher when compared to other parts of Africa. Also in these communities, marriages are more stable and there is minimal intermarriage between ethnic groups. In addition, there are areas where first cousin marriages still occurs for example, among the Fulani's, allowing for the detection of rare genetic defects and maybe a good source of material for genetic research.

Other polymorphisms at candidate gene loci that have proved to be interesting in this study in relation to TB may be analysed to further delineate their role in mycobacterial diseases.

For novel polymorphisms that might be functional judged by their being at or around putative transcription factor binding sites, assays to determine how alleles of these polymorphisms drive rate of gene expression may be carried out. Theses assays may range from carrying out electrophoretic mobility shift assay to show binding affinity of transcription factors for their binding sites, to transfection assays using gene construct to report differences in rate of gene transcription between wild-type and mutant allele.

Finally, all these mentioned above might be carried out in other mycobacterial diseases for example, leprosy caused by *Mycobacterium leprae.* Leprosy provides a unique opportunity to study genetic regulation of immune responses because of the ease of disease classification. It should be interesting to determine if the same genes are associated with the two human mycobacterial diseases in The Gambia. This should facilitate the development of prophylaxis required for the prevention of mycobacterial diseases.

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APPENDICES

APPENDIX 1 BUFFERS FOR OPTIMISING PCR CONDITIONS

BUFFER Number	pH	Tris-HCL (mM)	$MgCl_2$ (mM)	KCL (mM)	Tween-20	Trition-X	H2O
1	8.3	10 (1.0ml)	1.5 (150µl)	25 (2500µl)	0	0.1% (100µl)	6.35ml
2	8.3	10 (1.0ml)	1.5 (150µl)	75 (7500µl)	0	0.1% (100µl)	1.35ml
3	8.8	10 (1.0ml)	1.5 (150µl)	25 (2500µl)	0	0.1% (100µl)	6.35ml
4	8.8	10 (1.0ml)	1.5 (150µl)	75 (7500µl)	0	0.1% (100µl)	1.35ml
5	9.2	10 (1.0ml)	1.5 (150µl)	25 (2500ul)	0	0.1% (100µl)	6.35ml
6	9.2	10 (1.0ml)	1.5 (150µl)	75 (7500µl)	0	0.1% (100µl)	1.35ml
7	8.3	10 (1.0ml)	3.5 (350µl))	25 (2500µl)	0	0.1% (100µl)	6.10ml
8	8.3	10 (1.0ml)	3.5 (350µl)	75 (7500µl)	0	0.1% (100µl)	1.10ml
9	8.8	10 (1.0ml)	3.5 (350µl)	25 (2500µl)	0	0.1% (100µl)	6.10ml
10	8.8	10 (1.0ml)	3.5 (350µl)	75 (7500µl)	0	0.1% (100µl)	1.10ml
11	9.2	10 (1.0ml)	3.5 (350µl)	25 (2500µl)	0	0.1% (100µl)	6.10ml
12	9.2	10 (1.0ml)	3.5 (350µl)	75 (7500µl)	0	0.1% (100µl)	1.10ml
13	8.3	10 (1.0ml)	2.0 (200µl)	50 (5000µl)	0	0.1% (100µl)	3.7ml
14	8.8	10 (1.0ml)	2.0 (200µl)	50 (5000µl)	0	0.1% (100µl)	3.7ml
15	9.2	10 (1.0ml)	2.0 (200µl)	50 (5000µl)	0	0.1% (100µl)	3.7ml
16	8.8	67 (6.7ml)	2.0 (200µl)	$(NH_4)_2SO_4$ 16 (1600µl)	0.1% (100µl)	0	1.40ml

Buffers were made up in volumes of 10 mls and reagents were made up in 1M.stock. To make up 10 ml buffer solution. One ml of 1M stock of Tris –HCl_2 was used at 10ml to achieve a final concentration of 10 mM. One hundred and fifty (150 µl) or three hundred and fifty (350 µl) microliters of $MgCl_2$ was used to obtain a concentration of 1.5 mM and 3.5 mM of $MgCl_2$ respectively and 2,500 µl or 6,750 µl of KCL was used to obtain 25 mM or 75 mM of KCl.

APENDIX 2 Estimates of alleles of candidate genes or markers

Table 2.1) Estimates of alleles against band sizes in base pairs for microsatellite markers

ALLELES	IL-10	NRAMP1	IL-4	IL-9	TNF	IFNγR1	IFNγ	NOS2A	D17s250
1	255.5	122.5-123.5	170.6-177.2	124.5-125.5	98.5-99.5	187.2-188.2	143	177.6-180	150.6-151.8
2	257	120.5-121.5	177.9-179.1	126.5-127.5	100.2-101.4	189-190.4	145	183-184.6	152.2-153.81
3	259	118.5-119.5	179.8-180.8	128.7-129.7	102.5-103.5	191.4-192.4	147	187.4-190	154.6-156.0
4	261.5	109-111	181.6-182.8	130.7-131.7	104.5-105.5	193.4-194.4	149	191.4-194.6	158.6-160.0
5	263.5		183.7-184.7	132.8-133.8	106.6-107.2	195.5-196.5	151	197.4-199.8	160.0-162.0
6	265.5		185.6-186.8	135-136	108.2-109.5	197.7-198.5	153	203.0-204.8	162.8-164.2
7	266.5		187.4-188.6	137-138	110.5-111.6	199.7-200.7	155	208.4-210.4	164.6-166.2
8	268.5		189.4-190.6	139.4-140.4	112.3-113.4	201.9-202.9	157	213.8-216.2	166.6-168.2
9	270.5		191.2-192.4	141.7-142.7	114.2-115.4	204.1-205.1	159	218.8-220.4	168.6-170.2
10	273		193.6-194.2	122.5-123.5	116.2-117.4	206.1-207.1		223.6-225.8	146.6-147.6
11	274.5		195.0-196.2	143.9-144.9	118.4-119.5	208.1-209.1		172.3-175.3	144.0-145.0
12			197.0-198.2	146.2-147.2	120.5-121.5	210.3-211.3		228.3-231.3	135.0-136.0
13			198-8-200.0		122.5-123.5	212.5-213.5		233.5-235.9	171.0-172.0
14			200.8-202.0		124.5-125.5	214.6-215.6		172.6-175	173.0-174.0
15			202.9-204.1					166.8-170	139.0-140.0
16			204.8-206						148.0-149.6
17			206.6-207.8						137.0-138.0
18			208.4-209.6						142.0-143.0
19			210.2-210.6						
20			212.1-213.3						
21			213.9-215.1						

Table 2.2) Estimates of alleles against band sizes in base pairs for PCR RFLP

ALLELES	NRAMP1 274 C/T	NRAMP 1 INT4	NRAMP1 INT13	IL-1β-511	IL-1βα+3953	IL-9(T113M)	TNFα308	TNF-β	IFNγR1ddelTT
1	167, 37, 12	624	142, 75, 24	304	97, 85, 12	462	87, 20	740	311
2	102, 65, 37, 12	455, 169	102, 75, 40, 24	191, 113	182, 12	321, 141	107	555, 185	206, 105

Table 2.3) Estimates of alleles against band sizes in base pairs for ARMS PCR

Allele	TNF-α-238	TNF-α -376	TNF--α857	TNF-α863	control
Wild type	395	396	270	263	769
mutant	395	396	270	263	

Table 2.4) Estimates of alleles against band sizes in base pairs for PCR VNTR

Alleles	IL-1RA	IL-4P2
1	410	183
2	242	253
3	325	123
4	500	

APPENDIX 3 Optimisation of TNF levels in response to LAM and LPS

Table 3.1 TNF concentration induced by different levels of LAM in the presence and absence rIFNγ.

Lam conc	Personal Identification	LAM undiluted	LAM diluted	IFN/LAM undiluted	IFN/LAM diluted
1ng/ml	LAM/GC 001	1491	2910	570	1600
	LAM/GC 002	116	645	114	960
	LAM/GC 003	73	530	96	970
	LAM/GC 004	88	555	76.5	880
	LAM/GC 005	851	2340	335.5	2640
	LAM/GC 006	60.5	480	83.5	895
	LAM/GC 007	756	1885	283.5	1625
	LAM/GC 008	116.5	860	156.5	1535
10ng/ml	LAM/GC 001	1495.5	2880	613.5	1535
	LAM/GC 002	149.5	800	74.5	785
	LAM/GC 003	117	690	75.5	750
	LAM/GC 004	132	800	71.5	960
	LAM/GC 005	1016	2395	305.5	1760
	LAM/GC 006	117	615	65.5	720
	LAM/GC 007	1004.5	2165	172.5	1250
	LAM/GC 008	172.5	1115	110	1205
100ng/ml	LAM/GC 001	1407.5	2945	864.5	1770
	LAM/GC 002	127	830	324	1005
	LAM/GC 003	91.5	855	127	670
	LAM/GC 004	109.5	820	153	740
	LAM/GC 005	703.5	2260	392	1295
	LAM/GC 006	92	780	350	1005
	LAM/GC 007	938	2610	315	985

	LAM/GC 008	148.5	1185	143	845
1000ng/ml	LAM/GC 001	2072	5635	2195.5	7825
	LAM/GC 002	1193.5	3450	2020	4570
	LAM/GC 003	428	1150	1305.5	2850
	LAM/GC 004	398	1085	1399.5	4070
	LAM/GC 005	2218	1023.5	2323	13780
	LAM/GC 006	187.5	1150	1724	4415
	LAM/GC 007	1632.5	4750	1662	5510
	LAM/GC 008	206.5	1000	659.5	2190
2500ng/ml	LAM/GC 001	2090.5	6930	2150	10570
	LAM/GC 002	1398.5	3450	2164.5	10765
	LAM/GC 003	1055	2010	1857	5905
	LAM/GC 004	752	1595	1763.5	5770
	LAM/GC 005	2431.5	1490	2701.5	18870
	LAM/GC 006	263	870	2086	6845
	LAM/GC 007	2124	7555	2184	9335
	LAM/GC 008	366	982	1482.5	4900

Table 3.2 TNF concentration induced by different levels of LPS

STIMULANT		PERSONAL IDENTIFICATION										
IFN-γ U/ ml	LPS ng / ml	GC/001	GC/002	GC/003	GC/004	GC/005	GC/006	GC/007	GC/008	GC/009	GC/010	MEAN
0	0	106	276	103	58	49	76	228	110	130	48	118.4
50	0	185	919	40	24	22	234	190	122	168	244	214.8
100	0	260	1274	158	108	178	138	118	132	126	66	255.8
200	0	347	1071	136	110	266	54	141	132	158	50	246.5
400	0	501	1042	138	134	234	184	166	296	86	182	296.3
800	0	525	1092	282	194	226	330	192	162	162	54	321.9
0	0.1	1320	1410	275	285	520	2680	1855	812	1085	880	1112.2
50	0.1	1380	1950	405	131	405	605	2460	3465	2630	545	1397.6
100	0.1	1795	2150	455	460	1370	2330	1455	1535	3605	3020	1817.5
200	0.1	1980	2260	565	580	1075	3830	1815	1570	3230	3615	2052
400	0.1	1730	1890	675	395	1000	2135	3945	3245	3815	3355	2218.5
800	0.1	1890	1810	565	745	1395	4080	2120	3040	4460	1025	2113
0	1.0	2550	3700	530	880	1870	5450	3590	5750	9650	4310	3738
50	1.0	5800	5150	730	840	1360	5000	5240	8160	11370	4970	4862
100	1.0	7500	6150	1140	1210	2440	5990	4490	4640	13620	9390	5657
200	1.0	7550	6850	1010	1480	3390	6510	3190	5470	14420	1910	5178
400	1.0	7900	2950	1010	1170	2770	8410	4900	7740	16420	9710	6298
800	1.0	8600	4300	1160	1790	4220	11490	7740	7950	15230	1083	6356.3
0	10	5900	3750	840	1770	650	6690	5630	4880	15650	4040	4980
50	10	8050	4050	810	1710	1860	6690	6710	8140	12560	1030	5161
100	10	6400	4750	980	3800	2580	7050	3650	6680	15510	8470	5987
200	10	9400	5050	1180	3710	2900	9490	1940	5810	17620	10510	6761
400	10	7850	5000	1070	2310	3130	7460	7950	9630	20270	9530	7420
800	10	8900	4650	1320	3730	1940	11970	8580	10370	19270	8350	7908

Table 3.3.1 Effect of Co IFN-γ-incubation on TNF levels

PERSONAL IDENTIFICATION										
	GC/007		GC/003		GC/011		GC/012		MEAN	
INCUBATION TIME	LPS alone	LPS IFN-γ	LPS Alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ
3 hours	359	486	81	82	107	124	194	291	185.2	245.7
5 hours	537	978	158	223	246	333	371	804	303	584.5
8 hours	2500	4830	450	1170	860	1610	960	3270	1192.5	2720
18 hours	2970	7250	3020	2010	1270	2650	910	1020	2042.5	3232.5

Table 3.3.2 Effect of Pre IFN-γ incubation on TNF levels

PERSONAL IDENTIFICATION										
	GC/007		GC/003		GC/011		GC/012		MEAN	
INCUBATION TIME	LPS alone	LPS IFN-γ	LPS Alone	LPS IFN-γ	LPS Alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ
3 hours	474	1108	205	210	419	642	255	346	338.3	576.5
5 hours	701	1938	414	441	444	837	349	765	477	995.2
8 hours	2740	9230	810	500	850	2740	630	1300	1257.5	3442.5
18 hours	2280	14180	650	830	850	1900	370	1060	1037.5	4305.7

Table 3.4.1 Effect of 1 / 2 whole blood dilution on TNF levels

PERSONAL IDENTIFICATION												
	GC/013		GC/014		GC/015		GC/016		GC/017		GC/018	
INCUBATION TIME	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ
3 hours	421	1538	85	677	285	1162	330	1405	335	1356	332	1268
5 hours	706	2052	111	1317	456	2130	351	1883	417	1948	362	1771
8 hours	1610	5450	200	2640	1240	7160	750	370	850	4730	580	4290
18 hours	1280	5930	180	2400	1180	7270	550	4320	760	4950	480	3140

Table 3.4.2 Effect of 1 / 8 whole blood dilution on TNF levels

PERSONAL IDENTIFICATION												
	GC/013		GC/014		GC/015		GC/016		GC/017		GC/018	
INCUBATION TIME	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ	LPS alone	LPS IFN-γ
3 hours	389	1063	165	627	234	810	414	1116	378	1057	399	1294
5 hours	714	1319	228	1279	417	1698	868	1980	604	1950	637	2194
8 hours	2180	9150	580	4110	1970	8900	3050	9130	2300	8800	1690	8140
18 hours	4120	11510	710	5540	4070	13340	5190	12140	4270	11550	2310	9530

Recovered cases				Controls			
LPS	ln (LPS)	LPS +IFN	ln (LPS+IFN)	LPS	ln (LPS)	LPS +IFN	ln (LPS+IFN)
4390	8.387	9805	9.1906	1215	7.1025	4955	8.5082
6075	8.712	8470	9.0443	930	6.8352	4670	8.4489
3335	8.112	9900	9.2003	1210	7.0984	2470	7.8120
945	6.851	5245	8.5650	1605	7.3809	2105	7.6521
4125	8.325	7765	8.9574	695	6.5439	5735	8.6543
190	5.247	420	6.0403	685	6.5294	1395	7.2406
140	4.942	1265	7.1428	305	5.7203	3525	8.1676
210	5.347	2480	7.8160	615	6.4216	404	6.0014
600	6.397	2675	7.8917	1020	6.9276	6475	8.7757
1285	7.159	4815	8.4795	485	6.1841	4170	8.3357
490	6.194	840	6.7334	430	6.0638	1870	7.5337
735	6.600	1850	7.5229	2185	7.6894	3730	8.2242
335	5.814	895	6.7968	120	4.7875	405	6.0039
1466	7.290	3025	8.0147	976	6.8835	2340	7.7579
863	6.760	3553	8.1755	550	6.3099	2390	7.7790
886	6.787	4596	8.4329	546	6.3026	3380	8.1256
736	6.601	1660	7.4146	1330	7.1929	3030	8.0163
556	6.321	1860	7.5283	756	6.6280	1336	7.1974
680	6.522	2766	7.9252	666	6.5013	1300	7.1701
953	6.860	3690	8.2134	580	6.3630	3770	8.2348
5410	8.596	5290	8.5736	3770	8.2348	6020	8.7028
393	5.974	1206	7.0951	993	6.9007	1986	7.5939
470	6.153	1130	7.0300	4610	8.4360	8350	9.0300
3650	8.202	5500	8.6125	420	6.0403	3280	8.0956
2440	7.800	5070	8.5311	1456	7.2834	7920	8.9771
4096	8.318	8880	9.0916	2270	7.7275	13260	9.4925
1140	7.039	3346	8.1155	4750	8.4659	13760	9.5295
446	6.100	2760	7.9230	726	6.5876	5500	8.6125
1793	7.492	18560	9.8288	10580	9.2667	10400	9.2496
1696	7.436	7725	8.9522	483	6.1800	2430	7.7956
2296	7.739	15903	9.6743	1256	7.1357	9553	9.1646
17270	9.757	26563	10.1873	17270	9.7567	26563	10.1873
6903	8.840	26200	10.1735	3926	8.2754	11913	9.3854
6300	8.748	11493	9.3495	9283	9.1359	25320	10.1393
8805	9.083	7120	8.8707	2883	7.9666	3403	8.1324
9160	9.123	14646	9.5919	4286	8.3631	7896	8.9741
3416	8.136	8640	9.0642	4715	8.4585	12870	9.4627
776	6.654	1576	7.3626	7140	8.8735	12100	9.4010

Appendix 4.1

TNF levels in picograms/ml in matched recovered cases and controls.

Baseline TNF levels were subtracted to derive the totals, and natural logarithms calculated. The analysis of these data is shown in table 3.

	Recovered cases				Controls			
	LPS	ln (LPS)	LPS +IFN	ln (LPS+IFN)	LPS	ln (LPS)	LPS +IFN	ln (LPS+IFN)
1	2055	7.6280	1335	7.1967	483	6.1800	218	5.3845
2	1722	7.4512	1630	7.3963	376	5.9296	460	6.1312
3	1353	7.2101	1165	7.0605	248	5.5134	540	6.2916
4	253	5.5334	1070	6.9754	890	6.7912	308	5.7301
5	2503	7.8252	1590	7.3715	252	5.5294	510	6.2344
6	112	4.7185	182	5.2040	175	5.1648	255	5.5413
7	143	4.9628	362	5.8916	85	4.4427	307	5.7268
8	30	3.4012	62	4.1271	371	5.9162	712	6.5681
9	177	5.1761	362	5.8916	31	3.4340	313	5.7462
10	1152	7.0493	955	6.8617	313	5.7462	658	6.4892
11	1040	6.9470	863	6.7604	292	5.6768	350	5.8579
12	1040	6.9470	305	5.7203	4178	8.3376	4622	8.4386
13	598	6.3936	520	6.2538	46	3.8286	57	4.0431
14	193	5.2627	215	5.3706	250	5.5215	278	5.6276
15	105	4.6540	123	4.8122	285	5.6525	293	5.6802
16	112	4.7185	198	5.2883	72	4.2767	122	4.8040
17	68	4.2195	130	4.8675	372	5.9189	645	6.4693
18	127	4.8442	167	5.1180	162	5.0876	232	5.4467
19	97	4.5747	148	4.9972	232	5.4467	195	5.2730
20	377	5.9322	440	6.0868	213	5.3613	197	5.2832
21	3768	8.2343	2802	7.9381	1180	7.0733	607	6.4085
22	102	4.6250	138	4.9273	63	4.1431	103	4.6347
23	58	4.0604	63	4.1431	927	6.8320	837	6.7298
24	5610	8.6323	6233	8.7376	1670	7.4206	963	6.8701
25	2065	7.6329	2813	7.9420	925	6.8298	410	6.0162
26					95	4.5539	123	4.8122
27					3143	8.0529	2943	7.9872
28					6032	8.7048	4983	8.5138

Appendix 4.2

IL-1β levels in picograms/ml in matched recovered cases and controls.
Baseline IL-1β levels were subtracted to derive the totals, and natural logarithms calculated. The analysis of these data is shown in table 3.

	Recovered cases				Controls			
	LPS	ln (LPS)	LPS +IFN	ln (LPS+IFN)	LPS	ln (LPS)	LPS +IFN	ln (LPS+IFN)
1	1152	7.049	681	6.524	447	6.103	560	6.328
2	866	6.764	519	6.252	146	4.984	155	5.043
3	1293	7.165	488	6.190	821	6.711	597	6.392
4	284	5.649	158	5.063	198	5.288	187	5.231
5	965	6.872	216	5.375	170	5.136	164	5.100
6	163	5.094	154	5.037	151	5.017	149	5.004
7	516	6.246	280	5.635	233	5.451	104	4.644
8	46	3.829	36	3.584	226	5.421	123	4.812
9	142	4.956	133	4.890	256	5.545	52	3.951
10	326	5.787	135	4.905	260	5.561	107	4.673
11	391	5.969	166	5.112	260	5.561	761	6.635
12	599	6.395	166	5.112	950	6.856	32	3.466
13	394	5.976	132	4.883	69	4.234	137	4.920
14	191	5.252	69	4.234	462	6.136	48	3.871
15	85	4.443	46	3.829	64	4.159	30	3.401
16	153	5.030	103	4.635	32	3.466	265	5.580
17	74	4.304	30	3.401	317	5.759	110	4.700
18	143	4.963	53	3.970	304	5.717	48	3.871
19	130	4.868	100	4.605	104	4.644	403	5.999
20	2087	7.643	1504	7.316	906	6.809	44	3.784
21	152	5.024	83	4.419	96	4.564	219	5.389
22	133	4.890	134	4.898	253	5.533	143	4.963
23	66	4.190	65	4.174	374	5.924	51	3.932
24	501	6.217	288	5.663	56	4.025	161	5.081
25	336	5.817	257	5.549	239	5.476	180	5.193
26	705	6.558	378	5.935	208	5.338	127	4.844
27	141	4.949	75	4.317	378	5.935	61	4.111
28	81	4.394	75	4.317	120	4.787	75	4.317
29	639	6.460	407	6.009	222	5.403	51	3.932
30	491	6.196	192	5.257	68	4.220	48	3.871
31	275	5.617	87	4.466	74	4.304	264	5.576
32	2494	7.822	1494	7.309	1765	7.476	451	6.111
33	1071	6.976	228	5.429	1048	6.955	270	5.598
34	1135	7.034	743	6.611	279	5.631	73	4.290
35	262	5.568	152	5.024	111	4.710	56	4.025
36	271	5.602	103	4.635	69	4.234	80	4.382
37	153	5.030	85	4.443	186	5.226	78	4.357
38	1114	7.016	706	6.560	189	5.242		

Appendix 4.3

IL-10 levels in picograms/ml in matched recovered cases and controls.

Baseline TNF levels were subtracted to derive the totals, and natural logarithms calculated. The analysis of these data is shown in table 3.

APPENDIX 5 MICROSATELLITE GENES DATA AND INFLUENCE ON MICROBIAL INDUCED RESPONSES

χ^2 is the symbol for chi square, D.F. represents degrees of freedom and p is the p value (conditional probability). There was no quantitative trait measurements for LAM induced responses for those with the genotype 2.1. p-valves were compared between four genotypes for each cytokine and stimulant. None of the alleles of the promoter repeat were shown to influence cytokine production.

TABLE 5.1 IL-41

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	13.5	10	0.19
IL-1β		8.46	10	0.58
Il-10		8.2	10	0.6
TNF	LAM&IFN-γ	13.9	10	0.17
IL-1β		15.8	10	0.10
IL-10		8.05	10	0.62
TNF	LPS	22.3	10	0.013
IL-1β		12.3	10	0.26
IL-10		12.7	10	0.24
TNF	LPS&IFNγ	18.6	10	0.04
IL-1β		16.7	10	0.08
IL-10		26.9	10	0.002

p valves as compared between eleven genotypes for each cytokine and stimulant.

Alleles of IL-4P1marker were shown to influence LPS induced TNF-a secretion (p = 0.013). Priming with IFN-γ though significantly different between alleles did not enhance this effect (p =0.04).

TABLE 5.2 IL-9

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	6.0	10	0.8
IL-1β		3.3	10	0.97
Il-10		3.85	10	0.95
TNF	LAM&IFN-γ	9.57	10	0.47
IL-1β		1.74	10	0.99
IL-10		5.9	10	0.82
TNF	LPS	9.22	10	0.51
IL-1β		21.1	10	0.02
IL-10		6.9	10	0.73
TNF	LPS&IFNγ	11.6	10	0.31
IL-1β		11.0	10	0.35
IL-10		13.5	10	0.19

p valves as compared between eleven genotypes for each cytokine and stimulant.

IL-1β release after LPS stimulation differed significantly between IL-9 genotypes (p=0.02).

TABLE 5.3 TNFa

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	26.6	17	0.06
IL-1β		19	17	0.33
Il-10		21.5	17	0.2
TNF	LAM&IFN-γ	18.67	17	0.35
IL-1β		15.6	17	0.55
IL-10		26.8	17	0.06
TNF	LPS	9.46	17	0.92
IL-1β		13.1	17	0.73
IL-10		7.9	17	0.99
TNF	LPS&IFNγ	8.3	17	0.96
IL-1β		10.4	17	0.88
IL-10		10.3	17	0.89

p valves as compared between eighteen genotypes for each cytokine and stimulant.

TABLE 5.4 IFNγR1

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	18.4	23	0.73
IL-1β		16.3	23	0.84
Il-10		17.5	23	0.77
TNF	LAM&IFN-γ	21.7	23	0.54
IL-1β		19.6	23	0.66
IL-10		20	23	0.64
TNF	LPS	22.1	23	0.51
IL-1β		21.4	23	0.55
IL-10		22.5	23	0.49
TNF	LPS&IFNγ	20.4	23	0.62
IL-1β		20.2	23	0.63
IL-10		26.2	23	0.29

p valves as compared between twenty four genotypes for each cytokine and stimulant.

TABLE 5.5 NOS2A

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	27.5	20	0.2
IL-1β		31.5	20	0.05
Il-10		24.2	20	0.23
TNF	LAM&IFN-γ	20.9	20	0.39
IL-1β		26.1	20	0.16
IL-10		23	20	0.25
TNF	LPS	18.8	20	0.53
IL-1β		20.5	20	0.43
IL-10		20.5	20	0.43
TNF	LPS&IFNγ	21.1	20	0.39
IL-1β		22	20	0.34
IL-10		25	20	0.19

p valves as compared between twenty one genotypes for each cytokine and stimulant.

Whole blood secretion of IL-1β after stimulation with LAM alone differed significantly between NOS2A genotypes (p=0.05).

TABLE 5.6 D17S250

Cytokines	Stimulant	χ^2	DF	p-value
TNF	LAM	21.8	24	0.59
IL-1β		15.1	24	0.92
Il-10		14.75	24	0.93
TNF	LAM&IFN-γ	23.6	24	0.48
IL-1β		21.2	24	0.6
IL-10		15.7	24	0.89
TNF	LPS	21.6	24	0.6
IL-1β		24.8	24	0.41
IL-10		16.8	24	0.86
TNF	LPS&IFNγ	26.2	24	0.34
IL-1β		24.3	24	0.44
IL-10		19.6	24	0.72

p valves as compared between twenty five genotypes for each cytokine and stimulant.

Cytokine induced in response to LAM or LPS stimulation and cytokine induced in response to pre IFN-γ and LAM stimulation.